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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :  
C12P 21/02, C12Q 1/68, C12N 15/11

A1

(11) International Publication Number:

WO 99/43844

(43) International Publication Date: 2 September 1999 (02.09.99)

(21) International Application Number: PCT/US99/04323  
(22) International Filing Date: 26 February 1999 (26.02.99)

(30) Priority Data:  
09/032,684 27 February 1998 (27.02.98) US  
09/185,115 3 November 1998 (03.11.98) US  
09/197,889 23 November 1998 (23.11.98) US

(63) Related by Continuation (CON) or Continuation-in-Part  
(CIP) to Earlier Application 09/197,889 (CIP)  
US  
Filed on 23 November 1998 (23.11.98)

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(81) Designated States: AU, CA, JP, MX, US, European patent  
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT,  
LU, MC, NL, PT, SE).

Published  
With international search report.

(54) Title: RECIPROCAL SUBTRACTION DIFFERENTIAL DISPLAY

(57) Abstract

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: a) selecting a first and second nucleic acid sample; b) producing libraries for the first and second nucleic acid sample; c) performing reciprocal subtraction between the libraries to produce two subtracted libraries; d) amplifying the two subtracted libraries; and e) comparing the two amplified subtracted libraries to identify differentially expressed nucleic acids. Also, this invention provides the above-described method, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer. This invention also provides the above-described methods, wherein the comparing of step e comprises using a gel to separate the nucleic acids from both of the libraries. This invention provides the isolated nucleic acid identified by the above-described methods, wherein the nucleic acid was not previously known to be differentially expressed between the two samples.

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RECIPROCAL SUBTRACTION DIFFERENTIAL DISPLAY

5 This application is a continuation-in-part of U.S. Serial  
No. 09/197,889, filed November 23, 1998, which is a  
continuation-in-part of U.S. Serial Application No.  
09/185,115, filed November 3, 1998 which is a  
continuation-in-part of U.S. Serial Application No.  
10 09/032,684, filed February 27, 1998. The content of the  
above identified applications are hereby incorporated  
into this application by reference.

15 Throughout this application, various references are  
referred to within parentheses. Disclosures of these  
publications in their entireties are hereby incorporated  
by reference into this application to more fully describe  
the state of the art to which this invention pertains.

20 Background of the Invention

Changes in gene expression are important determinants of  
normal cellular physiology, including cell cycle  
regulation, differentiation and development, and they  
directly contribute to abnormal cellular physiology,  
25 including developmental anomalies, aberrant programs of  
differentiation and cancer (1-4). In these contexts,  
the identification, cloning and characterization of  
differentially expressed genes will provide relevant and  
important insights into the molecular determinants of  
30 processes such as growth, development, aging,  
differentiation and cancer. A number of procedures can  
be used to identify and clone differentially expressed  
genes. These include, subtractive hybridization (5-10),  
differential RNA display (DDRT-PCR) (3,4, 11,12), RNA  
35 fingerprinting by arbitrarily primed PCR (RAP-PCR)  
(13,14), representational difference analysis (RDA) (15),  
serial analysis of gene expression (SAGE) (16,17),  
electronic subtraction (18,19) and combinatorial gene  
matrix analyses (20).

Since first introduced by Liang and Pardee (11), DDRT-PCR has gained wide popularity in analyzing and cloning differentially expressed genes. In DDRT-PCR, total RNAs or mRNAs from two or more cell types (or cells grown  
5 under different conditions, cells representing different stages of development, cells treated with agents modifying cellular physiology, etc.) are reverse-transcribed with two-base-pair anchored oligo dT primers, which divide mRNA populations into 12 cDNA  
10 subgroups. Then, each cDNA subgroup is amplified by PCR with one of 20 arbitrary 10-mer 5' primers and a 3' anchored primer and the PCR-amplified cDNA fragments are resolved in DNA sequencing gels. The combinations of  
15 primers are designed not only to yield a detectable size and number of bands, but also to display nearly the complete repertoire of mRNA species.

DDRT-PCR is a powerful methodology in which a vast number of mRNA species (>20,000, if no redundancy occurs) can be  
20 analyzed with only a small quantity of RNA (about 5  $\mu$ g) (11). DDRT-PCR is often the method of choice when the RNA source is limiting, such as tissue biopsies. A direct advantage of DDRT-PCR is the ability to identify and isolate both up- and down-regulated differentially  
25 expressed genes in the same reaction. Furthermore, the DDRT-PCR technique permits the display of multiple samples in the same gel, which is useful in defining specific diagnostic alterations in RNA species and for temporally analyzing gene expression changes. However,  
30 the DDRT-PCR technique is not problem free. Difficulties encountered when using standard DDRT-PCR include, a high incidence of false positives and redundant gene identification, poor reproducibility, biased gene display and lack of functional information about the cloned cDNA.  
35 Furthermore, poor separation can mask differentially expressed genes of low abundance under the intense signals generated by highly expressed genes. The



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generation of false positives and redundancy can be highly problematic, resulting in an inordinate expenditure of resources to confirm appropriate differential expression and uniqueness of the isolated  
5 cDNAs. The cDNAs must be isolated from the gels in pure form (contamination of bands with multiple sequences complicates clone identification), reamplified, placed in an appropriate cloning vector, analyzed for authentic differential expression and finally sequenced. These  
10 limitations of the standard DDRT-PCR approaches emphasize the need for improvements in this procedure to more efficiently and selectively identify differentially expressed genes.

15 A number of modifications and improvements of the DDRT-PCR approach have been described (21-23). Single anchor or degenerate two-base anchor oligo dT primers can be used to streamline the massive numbers of reverse transcription and PCR reactions required for validation  
20 of cDNAs as well as to reduce false positives (24,25). Reproducibility can be improved by lengthening the arbitrary 5' primers to accommodate a convenient restriction site followed by two cycles of PCR with successive low- and high-stringency annealing  
25 temperatures (25,26). DDRT-PCR with inosine-containing 5' arbitrary primers can also increase reproducibility of this approach (27). However, since these modifications have only been analyzed using a subset of primers, further studies are necessary to validate these  
30 modifications of DDRT-PCR with additional primers and in several model systems.

In addition to genomic DNA contamination, mispriming, PCR artifacts, the high incidence of false positives and  
35 redundancy is also ascribed to poor separation between bands and the complexity of the templates amplified (28). Furthermore, poor separation can mask differentially

expressed genes of low abundance under the intense signals generated by highly expressed genes. By enriching for unique cDNAs and removing common ones, it should in principle be possible to enrich for low abundant gene products and significantly decrease the complexity of amplified sequences. In addition, the sequence bias of DDRT-PCR should also be reduced by decreasing template complexity. These assumptions serve as the basis for the development of reciprocal subtraction differential RNA display (RSDD).

Subtractive hybridization, in which hybridization between tester and driver is followed by selective removal of common gene products, enriches for unique gene products in the tester cDNA population and reduces the abundance of common cDNAs (9). A subtracted cDNA library can be analyzed to identify and clone differentially expressed genes by randomly picking colonies or by differential screening (29-31). Although subtractive hybridization has been successfully used to clone a number of differentially expressed genes (5-7,10), this approach is both labor-intensive and does not result in isolation of the full spectrum of genes displaying altered expression (9,18).

In principle, DDRT-PCR performed with subtracted RNA or cDNA samples represents a powerful strategy to clone up and down-regulated gene products. This approach should result in the enrichment of unique sequences and a reduction or elimination of common sequences. This scheme should also result in a consistent reduction in band complexity on a display gel, thereby permitting a clearer separation of cDNAs resulting in fewer false positive reactions. Additionally, it should be possible to use fewer primer sets for reverse transcription and PCR reactions to analyze the complete spectrum of differentially expressed genes. Of particular importance

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for gene identification and isolation, rare gene products that are masked by strong common gene products should be displayed by using subtraction hybridization in combination with DDRT-PCR. In addition, the DDRT-PCR approach with subtractive libraries could also prove valuable for efficiently screening subtracted cDNA libraries for differentially expressed genes. However, even though subtraction hybridization plus DDRT-PCR appears attractive for the reasons indicated above, a previous attempt to use this approach has proven of only marginal success in consistently reducing the complexity of the signals generated, compared with the standard DDRT-PCR scheme (32).

We presently describe a reciprocal subtraction differential RNA display (RSDD) approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes displaying anticipated differential expression.

20

Summary of the Invention

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second  
5 nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) performing reciprocal subtraction between the nucleic acid samples to produce two subtracted nucleic acid samples; (c) amplifying the two subtracted nucleic acid samples; and  
10 (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention also provides a method for identifying differentially expressed nucleic acids between two  
15 samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) amplifying the two nucleic acid samples; (c) performing reciprocal subtraction between the amplified nucleic acid samples to  
20 produce two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention further provides the above-described  
25 methods, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.

This invention further provides the above-described  
30 methods, wherein the first and second nucleic acid samples are obtained from cells from different tissue types.

Also, this invention provides the above-described  
35 methods, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer.

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In addition, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer.

- 5 This invention also provides the above-described methods, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the libraries.

- 10 This invention provides the isolated nucleic acid identified by the the above-described methods, wherein the nucleic acid was not previously known to be differentially expressed between the two samples.

### Brief Description of the Figures

#### Figure 1

Identification of differentially expressed sequence tags using reciprocal subtraction differential RNA display (RSDD). Left panel: differential RNA display pattern of conventional DDRT-PCR with RNA from E11 (C) and E11-NMT (T) cells and an RSDD analysis of reciprocally subtracted E11 minus E11-NMT (C/T) and E11-NMT minus E11 (T/C) cDNA libraries. Right panel: representative RSDD patterns using different sets of primers.

#### Figure 2

Reverse Northern analysis of differentially expressed sequence tags identified by reciprocal subtraction differential RNA display (RSDD). Differentially expressed sequence tags obtained from RSDD were dot-blotted onto Nylon membranes and probed with <sup>32</sup>P-cDNA reverse transcribed from RNA samples of E11 and E11-NMT cells.

#### Figure 3A

Differential expression of representative progression elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting. Northern blots of E11 and E11-NMT RNA samples were probed with radiolabeled (<sup>32</sup>P) expressed sequence tags identified by RSDD and reverse Northern blotting.

#### Figure 3B

Differential expression of representative progression elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting.

#### Figure 4

Differential expression of representative progression

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elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting. Northern blots of cells displaying various stages of transformation progression were probed with radiolabeled (<sup>32</sup>P) expressed sequence tags identified by RSDD and reverse Northern blotting. The cell types used include, Unprogressed E11 (-), CREFx E11-NMT F1 (-) and CREFx E11-NMT F2 (-) somatic cell hybrids, E11x E11-NMT A6 (-) somatic cell hybrid, E11x E11-NMT 3b (-) somatic cell hybrid, and E11-NMT Aza B1 (-) and E11-NMT Aza C1 (-) 5-azacytidine treated E11-NMT clones; and Progressed E11-NMT (+), CREFx E11-NMT R1 (+) and CREFx E11-NMT R2 (+) somatic cell hybrids, E11x E11-NMT A6TD (+) nude mouse tumor derived somatic cell hybrid, E11x E11-NMT IIa (+), E11-Ras R12 (+) a Ha-ras transformed E11 clone and E11-HPV E6/E7 (+) an E11 clone transformed by the E6 and E7 region of HPV-18.

20 Figure 5

cDNA fragment of PEGen 7 - 90% Homology to Human HPV16 E1BP. (Sequence ID No. 1)

Figure 6

25 cDNA fragment of PEGen 8 - Rat phosphofructose kinase C. (Sequence ID No. 2)

Figure 7

First (Sequence ID No. 3) and second (Sequence ID No. 4) cDNA fragments of PEGen 13.

Figure 8

cDNA fragment of PEGen 14. (Sequence ID No. 5)

35 Figure 9

cDNA fragment of PEGen 15. (Sequence ID No. 6)

-10-

Figure 10

cDNA fragment of PEGen 21 which has 94% homology to mouse  
FIN 14. (Sequence ID No. 7)

5 Figure 11

cDNA fragment of PEGen 24. (Sequence ID No. 8)

Figure 12

10 cDNA fragment of PEGen 26 - Rat poly ADP-ribose  
polymerase. (Sequence ID No. 9)

Figure 13

cDNA fragment of PEGen 28. (Sequence ID No. 10)

15 Figure 14

cDNA fragment of PEGen 42. (Sequence ID No. 11)

Figure 15

20 cDNA fragment of PEGen 43. (Sequence ID No. 12)

Figure 16

cDNA fragment of PEGen 44. (Sequence ID No. 13)

Figure 17

25 cDNA fragment of PEGen 48. (Sequence ID No. 14)

Figure 18

30 cDNA fragment of PSGen 1 which has 80% homology to *B.*  
*taurus* supervillin. (Sequence ID No. 15)

Figure 19

cDNA fragment of PSGen 2 which has 91% homology to human  
HTLV-1 Tax interacting protein. (Sequence ID No. 16)

35 Figure 20

cDNA fragment of PSGen 4 - Rat proteasome activator.  
(Sequence ID No. 17)



Figure 21

cDNA fragment of PSGen 10 - Rat Ferritin Heavy Chain.  
(Sequence ID No. 18)

5     Figure 22

cDNA fragment of PSGen 12.     (Sequence ID No. 19)

Figure 23

cDNA fragment of PSGen 13.     (Sequence ID No. 20)

10

Figure 24

cDNA fragment of PSGen 23.     (Sequence ID No. 21)

Figure 25

15     cDNA fragment of PSGen 24.     (Sequence ID No. 22)

Figure 26

cDNA fragment of PSGen 25.     (Sequence ID No. 23)

20     Figure 27

cDNA fragment of PSGen 26.

Figure 28

cDNA fragment of PSGen 27.

25

Figure 29

cDNA fragment of PSGen 28.

Figure 30

30     cDNA fragment of PSGen 29.

Figure 31

cDNA fragment of PEGen 32.

35

Figure 32

Schematic outline of the reciprocal differential RNA display (RSDD) protocol. This scheme incorporates three steps, reciprocal subtraction of cDNA libraries, differential display of *in vivo* excised cDNAs and expression analysis by reverse Northern and standard Northern blotting. For the present application of RSDD, reciprocal subtraction hybridization was performed using libraries constructed from E11 and E11-NMT cells, i.e., E11 minus E11-NMT and E11-NMT minus E11. Differentially expressed cDNAs identified on gels using differential RNA were isolated, reamplified and analyzed for expression by reverse Northern blotting. To confirm differential expression cDNAs were analyzed using Northern blotting.

Figure 33

Differential expression of representative progression elevated (PEGen) and progression suppressed genes (PSGen) identified by RSDD and reverse Northern blotting. Northern blots of E11 and E11-NMT RNA samples were probed with radiolabeled ( $^{32}\text{P}$ ) expressed sequence tags identified by RSDD and reverse Northern blotting. Equal loading of E11 and E11-NMT RNA is demonstrated by ethidium bromide (EtBr) staining.

Figure 34

Differential expression of representative PEGen and PSGen genes identified by RSDD and reverse Northern blotting in a large panel of rodent cells displaying differences in transformation progression. Northern blots of cells displaying various stages of transformation progression were probed with radiolabeled ( $^{32}\text{P}$ ) expressed sequence tags identified by RSDD and reverse Northern blotting. The cell types used include: Unprogressed E11 (-), CREF X E11-NMT F1 (-) and CREF X E11-NMT F2 (-) somatic cell hybrids, E11 X E11-NMT A6 (-) somatic cell hybrid, E11 X E11-NMT 3b (-) somatic cell hybrid, and E11-NMT AZA B1

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(-) and E11-NMT AZA C1 (-) 5-azacytidine-treated E11-NMT clones; and Progressed E11-NMT (+), CREF X E11-NMT R1 (+) and CREF X E11-NMT R2 (+) somatic cell hybrids, E11 X E11-NMT A6TD (+) nude mouse tumor derived somatic cell hybrid, E11 X E11-NMT IIa (+), E11-Ras R12 (+) and E11-HPV E6/E7 (+) an E11 clone transformed by the E6 and E7 region of HPV-18. Equal loading of RNAs is demonstrated by ethidium bromide (EtBr) staining.

10 Figure 35 A

PSGen 12 cDNA Sequence and PSGen 12 Protein Sequence

Figure 35 B

PSGen 13 cDNA Sequence and PSGen 13 Protein Sequence

15

Figure 35 C

PEGen 28 cDNA Sequence and PEGen 28 Protein Sequence

Figure 35D

20 PEGen 32 cDNA Sequence and PEGen 32 Protein Sequence

Figure 35 E

PEGen 42 cDNA Sequence and PEGen 42 Protein Sequence

25 Figure 35 F

PEGen 45 cDNA Sequence

Figure 35 G-1 and Figure 35 G-2

30 PEGen 50 cDNA Sequence which are different parts of the gene.

Figure 36

PSGen 27 - Novel

Detailed Description of the Invention

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second  
5 nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) performing reciprocal subtraction between the nucleic acid samples to produce two subtracted nucleic acid samples; (c)  
10 amplifying the two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

In an embodiment, the nucleic acid samples are mRNA or derived from mRNA. In another embodiment, the nucleic  
15 acid samples are total RNA. In another embodiment, the nucleic acid samples are cDNA. In another embodiment, the nucleic acid samples are a nucleic acid library.

In an embodiment, differentially expressed nucleic acids  
20 are expressed at different levels. In a further embodiment, one of the nucleic acids is not expressed. In a different embodiment, one of the nucleic acids is expressed in truncated form.

25 As used herein, reciprocal subtraction includes using nucleic acid sample A to subtract common nucleic acids from nucleic acid sample B (based on hybridization) and also using nucleic acid sample B to subtract common  
30 nucleic acids from nucleic sample A. In an embodiment, the complement of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the complement of nucleic acid sample B is used to subtract  
35 nucleic acids from nucleic acid sample A. In a further embodiment, the RNA of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the RNA of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A. In yet another

embodiment, the cDNA of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the cDNA of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A.

5

As used herein, methods of amplification include PCR and rolling circle replication.

10

A basic description of nucleic acid amplification is described in Mullis, U.S. Patent No. 4,683,202, which is incorporated herein by reference. The amplification reaction uses a template nucleic acid contained in a sample, two primer sequences and inducing agents. The extension product of one primer when hybridized to the

15

second primer becomes a template for the production of a complementary extension product and vice versa, and the process is repeated as often as is necessary to produce a detectable amount of the sequence.

20

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E.coli* DNA polymerase I, thermostable *Taq* DNA polymerase, Klenow

25

fragment of *E.coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase and other enzymes which will facilitate combination of the nucleotides in the proper manner to form amplification products. The oligonucleotide primers can

30

be synthesized by automated instruments sold by a variety of manufacturers or can be commercially prepared based upon the nucleic acid sequence of this invention.

35

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: a) selecting a first and second nucleic acid sample; b) producing libraries for the first

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and second nucleic acid sample; c) amplifying the two libraries; d) performing reciprocal subtraction between the amplified libraries to produce two subtracted libraries; and e) comparing the two subtracted libraries to identify differentially expressed nucleic acids.

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) amplifying the two nucleic acid samples; (c) performing reciprocal subtraction between the amplified nucleic acid samples to produce two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention also provides the above-described methods, wherein the two subtracted nucleic acid samples from step c are amplified prior to the comparing of step d.

This invention also provides the above-described methods, wherein the each of the nucleic acid samples comprises a library of nucleic acids.

This invention also provides the above-described methods, wherein the nucleic acid samples are obtained from total cellular RNA purified by hybridization with oligo (dT).

This invention also provides the above-described methods, wherein the nucleic acid samples are obtained from total RNA from E11 and E11-NMT cells.

E11 is an adenovirus-transformed rat embryo cell line that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reisolated in cell culture (E11-NMT).

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.

5

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells from different tissue types.

10

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells that differ in their exposure to external factors or in their gene expression.

15

In an embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cells that may have different levels of gene expression, wherein some genes may not be expressed at all. In

20

another embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cells that are likely to have different levels of gene expression, wherein some genes may not be expressed at all. In still another embodiment, cells that differ

25

in their exposure to external factors or in their gene expression includes any cell that has a phenotypically recognizable difference.

30

A short list of examples of cells that differ in their exposure to external factors or in their gene expression includes: cancerous versus normal cells, advanced cancer progression cells versus earlier cancer stage cells, diseased cells versus nondiseased cells, infected cells versus noninfected cells, later developmental stage cells versus earlier developmental stage cells, cells after DNA

35

damage versus cells before DNA damage, senescent cells versus younger cells, cells induced by growth factors

versus cells not induced by growth factors, cells in the process of neurodegeneration versus normal cells, and cells exposed to a chemotherapeutic agent versus normal cells.

5

As used herein, different tissues types include but are not limited to tissues containing: cells grown under or exposed to different conditions, cells in different stages of development, cells treated with agents  
10 modifying cellular physiology, and cells having different functions.

15

In an embodiment, cells at different stages of development are cells taken or analyzed at times differing by one or more hours in the development of the cell or organism.

20

Further, this invention provides the above-described methods, wherein the amplifying of step (d) comprises PCR amplification.

25

Also, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer. A few examples of oligo dT primers are T<sub>13</sub>, T<sub>13</sub>A, and T<sub>13</sub>GA.

30

In addition, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer. Oligo dT 3' primers include T<sub>13</sub>A, T<sub>13</sub>C, and T<sub>13</sub>G.

35

This invention provides the above-described methods, wherein the PCR amplification uses a set of random primers.

This invention provides the above-described methods, wherein the 5' primer is an arbitrary primer.



This invention also provides the above-described methods, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the subtracted libraries.

5

In an embodiment, the gel is a polyacrylamide gel. In another embodiment, the gel is an agarose gel.

10

This invention further provides the above-described methods, further comprising PCR amplifying the first and second nucleic acid samples.

15

This invention also provides the above-described methods, further comprising reamplifying differentially expressed bands.

20

This invention also provides the above-described methods, further comprising reamplifying differentially expressed nucleic acid.

25

In one method of reamplifying differentially expressed bands, differentially amplified bands from plasmids of each subtracted library were marked with an 18G needle through the film and cut out with a razor. The cut out differentially expressed bands can be reamplified (i.e. by PCR) and examined by reverse Northern and Northern blot analyses.

30

In addition, this invention provides the above-described methods, wherein the comparing of step (e) comprises comparing the band intensities of the two amplified differentially expressed nucleic acids.

35

In addition, this invention provides the above-described methods, wherein the nucleic acid samples are mRNA or cDNA derived from mRNA.

-20-

In addition, this invention provides the above-described methods, wherein the comparing of step (e) comprises comparing the quantities of the two amplified differentially expressed nucleic acids.

5

This invention further provides the above-described methods, wherein the differences in band intensity between the two subtracted libraries are electronically quantified.

10

This invention further provides the above-described methods, wherein the differences in the quantities of nucleic acid between the two subtracted libraries are electronically quantified.

15

In one embodiment, electronic quantification involves using a scanner to detect the bands. In a further embodiment, computer software, such as Corel Draw, can be used to determine the pixel intensity of the scanned image, thereby quantifying the band intensity.

20

Also, this invention provides the above-described methods, wherein the libraries of step (b) are constructed with  $\lambda$ -ZAP cDNA library kits. One skilled in the art would recognize that any cDNA library would be suitable.

25

This invention provides the isolated nucleic acid identified by the the above-described methods, wherein the nucleic acid was not previously known.

30

This invention also provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSCen 12 (AI 144569).

35

In addition, this invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid

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is the nucleic acid designated PSGen 13 (Accession No. AI 144570).

5 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 23.

10 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 24.

15 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 25.

20 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 26 (Accession No. AI 144571).

25 This invention also provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 27 (Accession No. AI 144572).

30 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 28 (AI 144573).

35 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 29 (AI 144574).

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 13 (AI 144564).

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This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 14 (AI 144565).

- 5 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 15.

- 10 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 24 (Accession No. AI 144566).

- 15 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 28 (AI 144567).

- 20 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 32 (AI 144568).

- 25 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 42.

- This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 43.

- 30 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 44.

- 35 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 48.

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This invention further provides a previously unknown isolated nucleic acid molecule identified by the above-described methods which comprises (a) one of the nucleic acid sequences as set forth in Figure 35; (b) a sequence  
5 being degenerated to a sequence of (a) as a result of the genetic code; (c) a sequence encoding one of the amino acid sequences as set forth in Figure 35. (d) a sequence of at least 12 nucleotides capable of specifically hybridizing to the sequence of (a), (b) or (c).

10

Finally, this invention provides a purified polypeptide comprising one of the amino acid sequence as set forth in Figure 35.

15 The sequences of the cDNA of PSGen 12, PSGen 13, PSGen 26, PSGen 27, PSGen 28, PSGen 29, PEGen 13, PEGen 14, PEGen 24, PEGen 28, and PEGen 32 were submitted to GenBank and assigned with accession numbers AI 144569, AI 144570, AI 144571, AI 144572, AI 144573, AI 144574, AI  
20 144564, AI 144565, AI 144566, AI 144567 and AI 144568 respectively.

This invention will be better understood from the Experimental Details which follow. However, one skilled  
25 in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

### 30 Experimental Details

We presently describe a reciprocal subtraction differential RNA display (RSDD) approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes  
35 displaying anticipated differential expression. Proof of principle for the RSDD approach has come from its application for the identification of genes

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differentially expressed during cancer progression. RSDD has resulted in the identification and cloning of genes displaying elevated expression in progressed tumor cells (PEGen) and reduced expression in progressed tumor cells (PSGen). The model used for RSDD was an adenovirus-transformed rat embryo cell line, E11, that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reisolated in cell culture (E11-NMT) (10,33,34). Injection of E11 cells into nude mice results in tumors in 100% of animals with a tumor latency time of approximately 35 to 40 days, whereas E11-NMT cells form tumors in 100% of nude mice with a tumor latency time of 15 to 20 days (10,34,35). Additionally, E11 cells form colonies in agar with an efficiency of ~3%, whereas E11-NMT display an agar cloning efficiency of >30% (10,33,34). The increased tumorigenicity and enhanced anchorage independence phenotypes are key indicators of tumor progression in the E11/E11-NMT model system (10,33,34).

Differential RNA display was directly performed with reciprocally subtracted cDNA plasmid libraries (E11 minus E11-NMT and E11-NMT minus E11). Compared with the subtraction of PCR-amplified cDNA in Hakvoort et al., the subtracted cDNA libraries used in this experiment are free from potential PCR artifacts and provide more stable and consistent sources for DDRT-PCR analyzes. In addition, three single anchored oligo dT 3' primers were used instead of two-base-anchored approach described by Hakvoort et al (32). To further streamline the DDRT-PCR procedure, reamplified cDNAs identified using RSDD were analyzed using the reverse Northern blotting procedure (35,36). cDNAs displaying differential expression by reverse Northern blotting were subsequently confirmed for true differential expression by Northern analysis. These modifications incorporated in the RSDD strategy result in an efficient approach for using subtractive hybridization

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and DDRT-PCR for identifying differentially expressed genes.

### Methods

5 Total RNA from E11 and E11-NMT cells was isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly A<sup>+</sup> RNA was purified with oligo(dT) cellulose chromatography (5). Two  $\lambda$ -ZAP cDNA libraries from E11 and  
10 E11-NMT mRNA's were constructed with  $\lambda$ -ZAP cDNA library Kits (Stratagene) following the manufacturer's protocol. Reciprocal subtraction between E11 and E11-NMT libraries was performed and two subtracted cDNA libraries (E11 minus E11-NMT and E11-NMT minus E11) were constructed as described previously. Bacterial plasmid libraries from  
15 the subtracted  $\lambda$ -ZAP cDNA libraries were obtained by *in vivo* excision following the manufacturer's protocol (Stratagene) and the plasmids were isolated with Qiagen columns (Qiagen Inc.).

20 The purified plasmids of reciprocally subtracted cDNA libraries were directly subjected to differential display as in Liang et. al. (38) with minor modifications. The plasmids of reciprocally subtracted cDNA libraries were PCR-amplified with the combination of three single-anchor  
25 3' primers (T<sub>13</sub> A, T<sub>13</sub> C or T<sub>13</sub> G) and 18 arbitrary 5' 10-mer primers obtained from Operon Technology Inc. (Alameda, CA. OPA 1-20 except OPA1 and 3). The 20  $\mu$ l PCR reaction consisted of 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2  $\mu$ M each dNTP, 0.2  $\mu$ M 5' arbitrary primer,  
30 1  $\mu$ M 3' anchor primer, 50 ng of plasmid of a subtracted library, 10  $\mu$ Ci  $\alpha$ -<sup>35</sup>S-dATP (3000 Ci/mmol from Amersham) and 1 U of Taq DNA polymerase (Gibco BRL). The parameters of PCR were 30 sec at 95 C, 40 cycle of 30 sec at 95 C, 2 min. at 40 C and 30 sec at 72 C and additional  
35 5 min. at 72 C. After the cycling, 10  $\mu$ l of 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol were added to each PCR reaction. The mixture was heated

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at 95 °C for 2 min and separated in a 5% denaturing DNA sequencing gel maintained at 50 °C. PCR reactions of plasmids from each subtracted library in a primer set were run side by side. Differentially amplified bands from plasmids of each subtracted library were marked with an 18G needle through the film and cut out with a razor. The gel slice was put in 100  $\mu$ l TE pH 8.0 and incubated at 4 °C overnight. After the incubation, the mixture was boiled for 5 min and microcentrifuged for two min. The supernatant was collected and stored at -20 °C until reamplification. The band extract was reamplified with the same cycling parameters in a 50  $\mu$ l reaction consisting of 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM  $MgCl_2$ , 20  $\mu$ M each dNTP, 0.2  $\mu$ M 5' arbitrary primer, 1  $\mu$ M 3' anchor primer, 5  $\mu$ l of band extract and 2.5 U of Taq DNA polymerase (Gibco BRL).

Differential expression of the reamplified DNA fragment was scrutinized by reverse Northern and Northern blot analyses. In reverse Northern analysis, after confirmation in a 1% agarose gel, the reamplified DNA fragment (10  $\mu$ l of PCR reaction) was mixed with 90  $\mu$ l TE and spotted on a positively charged Nylon membrane (Boehringer Mannheim) with a 96-well vacuum manifold. The membrane was soaked with denaturing and neutralizing solution successively, and the spotted DNA was crosslinked to the membrane with a UV crosslinker (Stratagene).  $^{32}P$ -labeled first strand cDNA was prepared by reverse transcription of total RNA. After heating at 70 °C for 10 min and quenching on ice for two min, 0.4  $\mu$ M each T<sub>13</sub>A, T<sub>13</sub>G and T<sub>13</sub>C and 10  $\mu$ g total RNA mixture was added with 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM  $MgCl_2$ , 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 0.5  $\mu$ l RNase inhibitor (Gibco BRL), 100  $\mu$ Ci dCTP (3000 Ci/mmmole from Amersham) and 200 U Superscript RT II (Gibco BRL) in a final 25  $\mu$ l reaction. The reaction mixture was incubated at 42 °C for one hr and at



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37 °C for 30 min after addition of 2 µl of RNase H (10U, Gibco BRL). The membrane was hybridized at 42 °C overnight in a 50% formamide hybridization solution. The hybridized membrane was washed at room temperature for 15 min with 2X SSC containing 0.1% SDS twice and at 55 °C for at least one hr with 0.1X SSC containing 0.1% SDS, successively. The membrane was probed with the <sup>32</sup>P-labeled cDNA of E11, stripped off and probed with <sup>32</sup>P-labeled cDNA of E11-NMT. The signal intensity of each spot was normalized against that of GAPDH and compared between E11 and E11-NMT. Reamplified DNA fragments displaying differential expression levels ≥1.8-fold higher between the two cell types were selected and analyzed by Northern blotting analysis.

In Northern blot analysis, 10 µg of total RNA from E11 and E11-NMT cells were run side-by-side in a 1% agarose gel with formaldehyde and transferred to a positively charged Nylon membrane. Reamplification reaction (5 µl) was <sup>32</sup>P-labeled with a multiprime labeling kit (Boehringer Mannheim) used to probe the membrane as described above. DNA fragments expressed differentially between E11 and E11-NMT in Northern blot analyses were cloned into the Eco RV site of the pZero-2.1 cloning vector (Invitrogene) and sequenced. In order to confirm differential expression, the cloned cDNA fragment was released by Eco RI -Xho I, <sup>32</sup>P-labeled and used to probe Northern blots as described above. Samples of RNAs from various E11 and E11-NMT derivatives displaying either a progressed or suppressed progression phenotype, based on nude mice tumorigenesis and soft agar cloning assays were analyzed. These included E11, E11-NMT, CREF X E11-NMT F1 and F2 somatic cell hybrids (suppressed progression phenotype), CREF X E11-NMT R1 and R2 somatic cell hybrids (progression phenotype), E11 X E11-NMT A6 somatic cell hybrid (suppressed progression phenotype), E11 X E11-NMT A6TD tumor-derived somatic cell hybrid (progression

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phenotype), E11 X E11-NMT 3b somatic cell hybrid (suppressed progression phenotype), E11 X E11-NMT 2a (progression phenotype), E11-NMT AZA B1 and C1 5-azacytidine treated E11-NMT clones (suppressed progression phenotype), E11-ras R12 clone containing the Ha-ras oncogene (progression phenotype) and E11-HPV E6/E7 clone containing the human papilloma virus-18 E6 and E7 gene region (progression phenotype). Differential expression of the PEGen and PSGen genes in the various cell types was confirmed using <sup>32</sup>P-labeled probes and Northern hybridization analysis. After reconfirmation of differential expression, the plasmids containing the differentially expressed DNA fragments were sequenced by the dideoxy sequencing procedure.

### Results and Discussion

Subtraction hybridization provides a direct means of enriching for unique cDNA species and eliminating common sequences between complex genomes. DDRT-PCR is a proven methodology for the rapid identification and cloning of differentially expressed sequences between cell types (3,4,22). In principle, subtraction hybridization combined with DDRT-PCR should reduce band complexity which often obscures the identification of differentially expressed genes and generates false positive signals (23,28). This strategy, RSDD, has been used to analyze genes differentially expressed during transformation progression. The differential RNA display pattern of E11 and E11-NMT cells using standard differential RNA display DDRT-PCR) and RSDD is shown in Fig. 1 (Left Panel). As predicted, the differential RNA display pattern of RSDD was much less complex than that of DDRT-PCR. The majority of bands common to both cDNA samples were eliminated using RSDD. These experiments demonstrate that subtractive hybridization prior to differential RNA display is effective in simplifying display patterns

permits the efficient identification of differentially expressed cDNAs. Since RSDD significantly reduced the number of bands displayed, single anchor oligo dT primers, that can increase band numbers, were successfully used in subsequent applications of the RSDD approach (Fig. 1; Right Panel). Using RSDD, 235 differentially displayed cDNAs in the E11/E11-NMT tumor progression model system were isolated.

10 Hakvoort et. al. (32) used a reciprocal subtraction approach to analyze gene expression changes resulting during liver regeneration following 70% hepatectomy, i.e., normal liver subtracted from partially hepatectomized regenerating liver and vice versa.

15 Although some bands displayed apparent enrichment, the complexity of the display pattern did not show appreciable simplification. These results are in stark contrast to RSDD, which results in a clear delineation and simplification of differentially expressed amplified

20 bands (Figs. 1). Although conceptually similar, RSDD is significantly more effective than the subtraction plus DDRT-PCR approach described by Hakvoort et al. (32). The improved efficiency of RSDD versus the Hakvoort et al. (32) approach can be attributed to several factors. The

25 approach of Hakvoort et al. (32) is based on the subtraction procedure described by Wang and Brown (38). This approach involves multiple rounds of PCR-amplification prior to each round of subtractive hybridization. In contrast, RSDD involves a single round

30 of reciprocal subtraction that does not involve PCR amplification (5,10). In this respect, the complicated display pattern observed by Hakvoort et al. (32) even after three or four rounds of subtraction might result from reduced subtraction efficiency, PCR artifacts or a

35 combination of these problems. Increasing the number of reactions by using two-base pair anchored oligo dT primers did not reduce the complexity of displayed bands

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(32). In these contexts, a critical component for the successful use of RSDD involves the use of an appropriate subtraction hybridization protocol, that can efficiently reduce cDNA complexity and generate stable populations of cDNAs for analysis.

Previous studies demonstrate that different gene cloning strategies, including DDRT-PCR, subtraction hybridization and electronic display, identify dissimilar differentially expressed genes (18). These results suggest that a single approach for gene identification may not identify the complete spectrum of differentially expressed genes (18). Similarly, RSDD and DDRT-PCR do not resolve the same differentially expressed bands (Fig. 1). Unique bands identified in DDRT-PCR that were differentially expressed when analyzed by Northern blotting were not the same as those found using RSDD and vice versa. These results are not surprising, since, as indicated above, subtraction hybridization and differential RNA display identified distinct differentially expressed genes. Apparently, specific differentially expressed genes are lost during subtraction hybridization and differential RNA display of subtracted cDNAs. On the basis of these considerations, it will be essential to use multiple gene discovery approaches to identify and clone the complete spectrum of differentially expressed genes.

DDRT-PCR can generate large numbers of differentially displayed bands making subsequent analysis both labor intensive and a daunting challenge. In order to reduce these limitations of DDRT-PCR, RSDD has been used in combination with reverse Northern analyses of isolated cDNAs. Gel extracted cDNA fragments were reamplified, dot-blotted on Nylon membranes and successively probed with reverse transcribed <sup>32</sup>P-cDNA from E11 or E11-NMT RNAs (Fig. 2). Signals were detected in 181 reamplified bands

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5 out of 235 (77%). This number is lower than that observed using DDRT-PCR (51 out of 54). However, this comparison may not be accurate since only four arbitrary primers were used for DDRT-PCR and fewer differentially expressed bands were detected and isolated. A possible reason for the high incidence of false positives in RSDD may be due to the existence of foreign plasmid-like DNA in the cDNAs and the inaccurate reading properties of DDRT-PCR.

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Table 1. Differentially Expressed cDNA Fragments  
Cloned by DDRT-PCR.

	Nomenclature	Identity	Homology
5	PEGen 41	To be determined	
	PEGen 42	Novel	Novel
10	PEGen 43	Novel	Novel
	PEGen 44	Novel	Novel
	PEGen 45	Hoxa11 locus antisense	mouse 90%
15	PEGen 46	Glutamyl t-RNA synthetase	human 59%
	PEGen 48	Novel	Novel
	PEGen 50	Novel	Novel
20	PSGen 1	Supervillin	B. <i>taurus</i> 80%
	PSGen 2	HTLV-1 Tax interacting protein	human 91%
	PSGen 4	Proteasome activator	Rat 100%
25	PSGen 27	Novel	

30 The signal intensities of the various cDNAs in reverse Northern analysis were quantified and normalized against that of GAPDH, which remained unchanged in E11 and E11-NMT cells. The PEG-3 (PEGen-3) gene (10) was used as an additional control, to verify increased expression in E11-NMT versus E11 cells. In the reverse Northern

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analyses, PEGen-3 levels were 4-fold higher in E11-NMT than in E11 cells, which coincided with Northern blotting results, thereby demonstrating the concordance of reverse Northern and Northern assays. A  $\geq 1.8$ -fold differential cut-off (after normalization for GAPDH expression) was used to identify and isolate cDNA bands displaying modified expression in E11 versus E11-NMT cells. This resulted in the identification of 7 cDNAs with higher expression in E11 versus E11-NMT cells and 65 cDNAs with elevated expression in E11-NMT versus E11 cells. These results suggest that tumor progression in E11-NMT cells correlates with the increased expression of a large number of genes, whereas only a smaller subset of genes display decreased expression.

A problem present in DDRT-PCR, that is reduced but still can occur in RSDD, is the isolation of multiple cDNA species from what appears to be a single amplified band. When this occurs, these multiple species can produce spurious results when analyzed by reverse Northern analyses. For example, if two distinct species are isolated, one displaying modified expression and a second not displaying modified expression, an accurate estimate of differential expression will not be obtained by reverse Northern analysis. In this case, a number of potential false positives generated using reverse Northern analyses, may in reality not be false positives, but instead may represent multiple cDNAs. This problem may be ameliorated by performing single strand conformational polymorphism (SSCP) or reverse Northern analyses using cloned cDNA populations (39,40).

The expression pattern of representative RSDD-derived cDNAs in E11 versus E11-NMT and in a more expanded E11/E11-NMT progression cell culture series is shown in Figs. 3 and 4, respectively. Reverse Northern results correlated well with Northern blots using E11 and

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E11-NMT (~80% concordance) or a larger panel of cells differentially displaying the progression phenotype, including progression negative, E11, CREF x E11-NMT F1, CREF X E11-NMT F2, E11 X E11-NMT A6, E11 X E11-NMT 3b, 5 E11-NMT Aza B1 and E11-NMT Aza C1, and progression positive E11-NMT, CREF X E11-NMT R1, CREF X E11-NMT R2, E11 X E11-NMT A6TD, E11 X E11-NMT IIa, E11-ras and E11-HPV E6/E7. Sequence analysis of the various progression upregulated genes (PEGen) and progression 10 suppressed genes (PSGen) identified both known and unknown genes (Table 2). Known PEGen genes included PEGen 7 (HPV16 E1BP), PEGen 8 (PFK-C), PEGen 21 (FIN 14) and PEGen 26 (poly ADP-ribose polymerase) and a known PSGen gene was PSGen 10 (ferritin heavy chain). Two 15 PEGen genes out of six were found to be novel (PEGen 14 and PEGen 24) and two PSGen genes out of three were found to be novel (PSGen 12 and PSGen 13) (Table 2).



Table 2. Differentially Expressed cDNA Fragments  
Cloned by RSDD

5			
	Nomenclature	Identity	Homology
	PEGen 7	HPV16 E1BP	Human 90%
	PEGen 8	PFK-C	Rat 100%
10	PEGen 13	Novel	Novel
	PEGen 14	Novel	Novel
	PEGen 15	Novel	Novel
15	PEGen 21	FIN 14	Mouse 94%
	PEGen 24	Novel	Novel
20	PEGen 26	Poly ADP-ribose Polymerase	Rat 100%
	PEGen 28	Novel	Novel
	PEGen 32	Novel	Novel
25	PSGen 10	Ferritin Heavy Chain	Rat 100%
	PSGen 12	Novel	Novel
30	PSGen 13	Novel	Novel
	PSGen 23	Novel	Novel

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	PSGen 24	Novel	Novel
	PSGen 25	Novel	Novel
5	PSGen 26	Novel	Novel
	PSGen 27	Novel	Novel
10	PSGen 28	Novel	Novel
	PSGen 29	Novel	Novel

15 PEGen 7 is expressed at ~ 5-fold higher levels in E11-NMT  
than in E11 cells. PEGen 7 is ~90% homologous to  
16E1-BP, a cDNA encoding a protein identified using the  
yeast two-hybrid assay that interacts with human  
papillomavirus type 16 E1 protein (41). 16E1-BP encodes  
20 a 432aa protein of unknown function but does contain an  
ATPase signature motif (Gly-X<sub>4</sub>-Gly consensus ATP binding  
motif at aa 179 through 186). 16E1-BP appears to be a  
form of TRIP13, a protein previously shown to bind  
thyroid hormone receptor in yeast two-hybrid assays. The  
25 role of PEGen 7/16E1-BP in the progression phenotype in  
the E11/E11-NMT progression model is not known.  
Additional studies are necessary to determine if this  
gene change is associative or causative of transformation  
progression.

30 PEGen 8 is expressed at ~3- to 4- fold higher levels in  
E11-NMT than in E11 cells. PEGen 8 shows 100% homology  
to rat phosphofructokinase C (PFK-C) (42). PFK catalyzes  
the rate-limiting and committed step in glycolysis, the  
35 conversion of fructose 6-phosphate to fructose  
1,6-biphosphate. Three subunit isozymes of PFK have been  
identified, that form homo- and heterotetramers with  
differing catalytic and allosteric properties. PFK-M is

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specific for cardiac and skeletal muscle, PFK-L is expressed in many tissues but is most abundant in the liver and PFK-C is expressed in several brain regions and the anterior pituitary but not in liver, skeletal muscle, or several other human tissues. The cDNA of PFK-C isolated from a rat hypothalamic cDNA library is 2643 bp and encodes a protein of 765aa (42). In a recent study, Sanchez-Martinez and Aragon (43) demonstrated that PFK-C is the predominant form of PFK in ascites tumor cells (obtained from a transplantable mouse carcinoma of mammary origin), whereas PFK-L is most abundant in the normal mammary gland. These results suggest the interesting possibility that PFK-C might contribute to the malignant nature of specific target cells. The role of PEGen 8/PFK-C in progression in the E11/E11-NMT model remains to be determined.

PEGen 21 is expressed at ~3- to 4-fold higher levels in E11-NMT than in E11 cells. PEGen 21 displays ~94% homology with the fibroblast growth factor-4 inducible gene FIN-14 (44). FIN-14 is a novel cDNA of unknown function that hybridizes with a 4.5 kb mRNA that is induced 4-fold in NIH3T3 mouse cells following treatment with FGF-4. The induction of FIN-14 occurs late (18 hr) after treatment with FGF-4 and does not occur when cells are treated for 18 hr with FGF-4 in the presence of cycloheximide (44). These results confirm that FIN-14 encodes a late-inducible gene. Moreover, nuclear run-on assays document that FIN-14 is transcriptionally activated in NIH3T3 cells following growth factor stimulation. Tissue distribution studies indicate expression of a single mRNA species in the kidney with low levels of expression observed in several other tissues including testis and thymus. Mouse embryogenesis studies indicate that FIN-14 expression occurs constitutively in mouse embryos between day 10.5 and 15.5. Unlike NIH3T3, FIN-14 was constitutively expressed in PC12 cells and its level

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did not vary appreciably in response to growth factor stimulation. The role of PEGen 21/FIN-14 in progression in E11/E11-NMT model system is not currently known.

5 The PGen cDNAs, PGen-12 and PGen-13, consist of sequences without homology to those presently reported in various DNA databases. Expression of these cDNAs is ~3- to 4-fold higher in E11 versus E11-NMT cells (Fig. 3). It is not currently known whether these genes simply  
10 correlate with or functionally regulate the progression phenotype. The identification of full-length cDNAs for PGen-12 and PGen-13 are in progress and once identified experiments can be conducted to directly define the role of these PGen's in cancer progression.

15 We presently demonstrate that a modified differential RNA display technique, RSDD, can efficiently identify differentially expressed cDNAs. As predicted, subtractive hybridization prior to differential RNA  
20 display greatly reduces band complexity, a problem encountered in standard DDRT-PCR in which RNA samples are directly analyzed without subtraction. Unlike a previous report using subtracted cDNAs processed through successive rounds of PCR (32,45), common bands were  
25 eliminated using reciprocally subtracted cDNA libraries that had not been processed using PCR. In addition to subtraction hybridization, the discovery of differentially expressed genes was further streamlined by using reverse Northern analyses with isolated cDNAs.  
30 With 3 single anchored oligo dT primers and 18 arbitrary 5' primers, 72 bands were identified that displayed differential expression using reverse Northern analysis. Currently, 40 of these cDNA species have been analyzed by Northern blotting and found to display differential  
35 expression in E11 versus E11-NMT cells. Subsequent studies with the majority of these RSDD cDNAs demonstrated coordinated expression with the progression

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phenotype in a large panel of unprogressed and progressed transformed cells. Current sequence analysis of the cloned cDNA fragments revealed 9 different genes, including 4 novel genes not reported in recent DNA  
5 databases. RSDD represents a method of choice either as a more efficient and less time consuming modification of the differential RNA display strategy or as a screening methodology for identifying differentially expressed genes in reciprocally subtracted cDNA libraries.

10

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### Second Series of Experiments

Presently described is a RSDD approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes displaying anticipated differential expression. The model used for RSDD was an adenovirus-transformed rat embryo cell line, E11, that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reestablished in cell culture (E11-NMT) (6,26,27). Injection of E11 cells into nude mice results in tumors in 100% of animals with a tumor latency time of approximately 35 to 40 days, whereas E11-NMT cells form tumors in 100% of nude mice with a tumor latency time of 15 to 20 days (6,26,27). Additionally, E11 cells form colonies in agar with an efficiency of ~3 %, whereas E11-NMT display an agar cloning efficiency of >30% (6,26,27). The increased tumorigenicity and enhanced anchorage independence phenotypes are key indicators of tumor progression in the E11/E11-NMT model system (6,26,27). RSDD has resulted in the identification and cloning of genes displaying elevated expression in progressed tumor cells (progression elevated gene, PEGen) and suppressed expression in progressed tumor cells (progression suppressed gene, PSGen).

### MATERIALS AND METHODS

**RNA isolation and cDNA library construction.** Total RNA from E11 and E11-NMT cells was isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly(A)<sup>+</sup> RNA was purified with oligo(dT) cellulose chromatography(5). Two  $\lambda$ -ZAP cDNA libraries from E11 and E11-NMT mRNAs were constructed with  $\lambda$ -ZAP cDNA library kits (Stratagene) following the manufacturer's protocol. Reciprocal subtraction between E11 and E11-NMT libraries was performed and two subtracted cDNA libraries (E11 minus E11-NMT and E11-NMT minus E11) were constructed as

described(5,6). Plasmid cDNA libraries from the subtracted  $\lambda$ -ZAP cDNA libraries were obtained by in vivo excision following the manufacturer's protocol (Stratagene) and the plasmids were isolated with Qiagen columns (Qiagen, Chatsworth, CA.).

**RSDD methodology.** The purified plasmids of reciprocally subtracted cDNA libraries were directly subjected to differential display as in Liang et al. (28) with minor modifications. The plasmids of reciprocally subtracted cDNA libraries were PCR-amplified with the combination of three single-anchor 3' primers ( $T_{13}A$ ,  $T_{13}C$  or  $T_{13}G$ ) and 18 arbitrary 5' 10-mer primers obtained from Operon Technology Inc. (Alameda, CA. OPA 1-20 except OPA1 and 3). The 20  $\mu$ l PCR reaction consisted of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM  $MgCl_2$ , 2  $\mu$ M each dNTP, 0.2  $\mu$ M 5' arbitrary primer, 1  $\mu$ M 3' anchor primer, 50 ng of plasmid of a subtracted library, 10  $\mu$ Ci  $\alpha$ - $^{35}S$ -dATP (3,000 Ci/mmol from Amersham) and 1 unit of Taq DNA polymerase (Gibco/BRL). The parameters of PCR were 30 sec at 95°C, 40 cycles of 30 sec at 95°C, 2 min at 40°C and 30 sec at 72°C and additional 5 min. at 72°C. After the cycling, 10  $\mu$ l of 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol were added to each PCR reaction. The mixture was heated at 95°C for 2 min and separated in a 5% denaturing DNA sequencing gel maintained at 50°C. PCR reactions of plasmids from each subtracted library in a primer set were run side by side. Differentially amplified bands from plasmids of each subtracted library were marked with 18G needle through the film and cut out with a razor. The gel slice was put in 100  $\mu$ l TE (pH 8.0) and incubated at 4°C overnight. After the incubation, the mixture was boiled for 5 min and microcentrifuged for two min. The supernatant was collected and stored at -20°C until reamplification. The band extract was reamplified with the same cycling parameters in a 50  $\mu$ l reaction consisting of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM

MgCl<sub>2</sub>, 20  $\mu$ M each dNTP, 0.2  $\mu$ M 5' arbitrary primer, 1  $\mu$ M 3' anchor primer, 5  $\mu$ l of band extract and 2.5 units of Taq DNA polymerase (Gibco/BRL).

5     **Reverse Northern Blotting Procedure.** Differential expression of the reamplified DNA fragment was scrutinized by reverse Northern and Northern blot analyses. In reverse Northern analysis, after confirmation in a 1% agarose gel, the reamplified DNA  
10     fragment (10  $\mu$ l of PCR reaction) was mixed with 90  $\mu$ l TE and spotted on a positively charged Nylon membrane (Boehringer Mannheim) with a 96-well vacuum manifold. The membrane was soaked with denaturing and neutralizing solution successively, and the spotted DNA was  
15     crosslinked to the membrane with a UV crosslinker (Stratagene). <sup>32</sup>P-labeled first strand cDNA was prepared by reverse transcription of total RNA. After heating at 70°C for 10 min and quenching on ice for two min, 0.4  $\mu$ M each T<sub>13</sub>A, T<sub>13</sub>G and T<sub>3</sub>C and 10  $\mu$ g total RNA mixture was  
20     added with 50 mM Tris-HCl, (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 0.5  $\mu$ l RNase inhibitor (Gibco/BRL), 100  $\mu$ Ci dCTP (3,000 Ci/mmol from Amersham) and 200 units Superscript RT II (Gibco/BRL) in a final 25  $\mu$ l reaction.  
25     The reaction mixture was incubated at 42°C for one hour and at 37°C for 30 min after addition of 2  $\mu$ l of RNase H (10 units, Gibco/BRL). The membrane was hybridized at 42°C overnight in a 50% formamide hybridization solution. The hybridized membrane was washed at room temperature for 15  
30     min with 2X standard saline citrate containing 0.1% SDS twice and at 55°C for at least one hour with 0.1X Standard Saline Citrate containing 0.1% SDS, successively. The membrane was probed with the <sup>32</sup>P-labeled cDNA of E11, striped off and probed with <sup>32</sup>P-labeled cDNA of E11-NMT.  
35     The signal intensity of each spot was normalized against that of glyceraldehyde-3-phosphate dehydrogenase and compared between E11 and E11-NMT. Reamplified DNA

fragments displaying differential expression levels  $\geq 1.8$ -fold higher between the two cell types were selected and analyzed by Northern blotting analysis.

5     **Northern Blotting Analysis.** In Northern blot analysis, 10  $\mu$ g of total RNA from E11 and E11-NMT cells were run side-by-side in a 1% agarose gel with formaldehyde and transferred to a positively charged Nylon membrane. Reamplification reaction (5  $\mu$ l) was  $^{32}$ P-labeled with a  
10     multiprime labeling kit (Boehringer Mannheim) used to probe the membrane as described above. DNA fragments expressed differentially between E11 and E11-NMT in Northern blot analyses were cloned into the EcoRV site of the pZero-2.1 cloning vector (Invitrogene) and sequenced.

15     To confirm differential expression, the cloned cDNA fragment was released by EcoRI-XhoI,  $^{32}$ P-labeled and used to probe Northern blots as described above. Samples of RNAs from various E11 and E11-NMT derivatives displaying  
20     either a progressed or suppressed progression phenotype, based on nude mice tumorigenesis and soft agar cloning assays were analyzed. These included E11, E11-NMT, CREF x E11-NMT F1 and F2 somatic cell hybrids (suppressed progression phenotype), CREF x E11-NMT R1 and R2 somatic  
25     cell hybrids (progression phenotype), E11 x E11-NMT A6 somatic cell hybrid (suppressed progression phenotype), E11 x E11-NMT A6TD tumor-derived somatic cell hybrid (progression phenotype), E11 x E11-NMT 3b somatic cell hybrid (suppressed progression phenotype), E11 x E11-NMT  
30     IIa (progression phenotype), E11-NMT AZA B1 and C1 5-azacytidine treated E11-NMT clones (suppressed progression phenotype), E11-Ras R12 clone containing the Ha-ras oncogene (progression phenotype) and E11-HPV E6/E7 clone containing the human papilloma virus-18 E6 and E7  
35     gene region (progression phenotype). Differential expression of the PEGen and PSGen genes in the various cell types was confirmed using  $^{32}$ P-labeled probes and



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northern hybridization analysis. After reconfirmation of differential expression, the plasmids containing the differentially expressed DNA fragments were sequenced by the dideoxy sequencing procedure.

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### RESULTS AND DISCUSSION

Subtraction hybridization provides a direct means of enriching for unique cDNA species and eliminating common sequences between complex genomes(7,18). DDRT-PCR is a proven methodology for the rapid identification and cloning of differentially expressed sequences between cell types (1,2,28). In principle, subtraction hybridization combined with DDRT-PCR should reduce band complexity which often obscures the identification of differentially expressed genes and generates false positive signals (21,29). RSDD has been used to analyze genes differentially expressed during transformation progression (Fig. 28). Differential RNA display was directly performed with reciprocally subtracted cDNA plasmid libraries (E11 minus E11-NMT and E11-NMT minus E11) that had not been subjected to PCR. Three single anchored oligo dT 3' primers were used for subsequent amplification prior to display. To further streamline the DDRT-PCR procedure, reamplified cDNAs identified using RSDD were analyzed using the reverse Northern blotting procedure (30,31). cDNAs displaying differential expression by reverse Northern blotting were subsequently confirmed for true differential expression by Northern analysis.

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The differential RNA display pattern of E11 and E11-NMT cells using standard differential RNA display (DDRT-PCR) and RSDD is shown in Fig. 1 (Left Panel). The differential RNA display pattern of RSDD is much less complex than that of DDRT-PCR. These experiments demonstrate that subtractive hybridization prior to differential RNA display is effective in simplifying

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display patterns permitting the efficient identification of differentially expressed cDNAs. Since RSDD significantly reduced the number of bands displayed, single anchor oligo dT primers, that can increase band numbers, were successfully used in subsequent applications of the RSDD approach (Fig. 1; Right Panel). Using RSDD, 234 differentially displayed cDNAs in the E11/E11-NMT tumor progression model system were isolated. Hakvoort et al. (25) used a reciprocal subtraction approach to analyze gene expression changes resulting during liver regeneration following 70% hepatectomy, i.e., normal liver subtracted from partially hepatectomized regenerating liver and vice versa. Although some bands displayed apparent enrichment, the complexity of the display pattern did not show appreciable simplification. In contrast, RSDD results in a clearer delineation and simplification of differentially expressed amplified bands (Figs. 1). Although conceptually similar, RSDD is significantly more effective than the subtraction plus DDRT-PCR approach described by Hakvoort et al. (25). The reasons for the improved efficiency of RSDD versus the Hakvoort et al. (25) approach are not known. One possibility is that the differences between the experimental approaches may reflect the subtraction hybridization strategies employed. The approach of Hakvoort et al. (25) is based on the subtraction procedure described by Wang and Brown (32). This approach uses multiple rounds of PCR-amplification prior to each round of subtractive hybridization. In contrast, RSDD involves a single round of reciprocal subtraction without intermediate amplification (5,6). In this respect, the complicated display pattern observed by Hakvoort et al. (25) even after three or four rounds of subtraction might result from reduced subtraction efficiency, PCR artifacts or a combination of these problems. Increasing the number of reactions by using two-base pair anchored oligo dT

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primers did not reduce the complexity of displayed bands (25). In these contexts, a critical component for the successful use of RSDD involves the use of an appropriate subtraction hybridization protocol, which can efficiently reduce cDNA complexity and generate stable populations of cDNAs for analysis.

Previous studies demonstrate that different gene cloning strategies, including DDRT-PCR, subtraction hybridization and electronic display, identify distinct subsets of differentially expressed genes (18). These results suggest that a single approach for gene identification may not identify the complete spectrum of differentially expressed genes. Similarly, RSDD and DDRT-PCR do not resolve the same differentially expressed bands (Fig. 1). Unique bands identified in DDRT-PCR that were differentially expressed when analyzed by Northern blotting were not the same as those found using RSDD and vice versa (data not shown). These results are not surprising, since, as indicated above, subtraction hybridization and differential RNA display identified distinct differentially expressed genes (18). Apparently, specific differentially expressed genes are lost during subtraction hybridization and differential RNA display of subtracted cDNAs. On the basis of these considerations, it will be essential to use multiple gene discovery approaches to identify and clone the complete spectrum of differentially expressed genes.

DDRT-PCR can generate large numbers of differentially displayed bands making subsequent analysis both labor intensive and a daunting challenge. In order to reduce these limitations of DDRT-PCR, RSDD has been used in combination with reverse Northern analyses of isolated cDNAs. Gel extracted cDNA fragments were reamplified, dot-blotted on Nylon membranes and successively probed with reverse transcribed <sup>32</sup>P-cDNA from E11 or E11-NMT RNAs

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(Fig. 2). Signals were detected in 181 reamplified bands out of 234 (77%).

The signal intensities of the various cDNAs in reverse Northern analysis were quantified and normalized against that of GAPDH, which remained unchanged in E11 and E11-NMT cells. Progression elevated gene-3 (PEG-3) (6) was used as an additional control, to verify increased expression in E11-NMT versus E11 cells. In the reverse Northern analyses, PEG-3 levels were 4-fold higher in E11-NMT than in E11 cells, which coincided with Northern blotting results, thereby demonstrating the concordance of reverse Northern and Northern assays. A  $\geq 1.8$ -fold differential cut-off (after normalization for GAPDH expression) was used to identify and isolate cDNA bands displaying modified expression in E11 versus E11-NMT cells. This resulted in the identification of 7 cDNAs with higher expression in E11 versus E11-NMT cells and 65 cDNAs with elevated expression in E11-NMT versus E11 cells. These results suggest that tumor progression in E11-NMT cells correlates with increased expression of a large number of genes, whereas only a smaller subset of genes display decreased expression.

A problem frequently encountered in DDRT-PCR, that is reduced but still can occur in RSDD, is the isolation of multiple cDNA species from what appears to be a single amplified band. When this occurs, these multiple species can produce spurious results when analyzed by reverse Northern analyses. For example, if two distinct species are isolated, one displaying modified expression and a second not displaying modified expression, an accurate estimate of differential expression will not be obtained by reverse Northern analysis. In this case, a number of potential false positives generated using reverse Northern analyses, may in reality not be false positives, but instead may represent multiple cDNAs. By performing

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single strand conformational polymorphism (SSCP) or reverse Northern analyses using cloned cDNA populations (33,34) this problem can be ameliorated.

5 The expression pattern of representative RSDD-derived cDNAs in E11 versus E11-NMT and in a more expanded E11/E11-NMT progression cell culture series is shown in Figs. 29 and 30, respectively. Reverse Northern results correlated well with Northern blots using E11 and E11-NMT  
10 (~75% concordance) or a larger panel of cells differentially displaying the progression phenotype, including progression negative E11, CREF x E11-NMT F1 and F2, E11 x E11-NMT A6, E11 x E11-NMT 3b, E11-NMT Aza B1 and Aza C1 cells, and progression positive E11-NMT, CREF  
15 x E11-NMT R1 and R2, E11 x E11-NMT A6TD, E11 x E11-NMT IIa, E11-Ras R12 and E11-HPV E6/E7 cells. Sequence analysis of the various PEGen cDNAs identified both unknown and known genes (Table 3). Five of 10 PEGen cDNAs  
20 (50%) were classified as novel sequences since no matches were found in current DNA databases. Novel PEGen cDNAs include, PEGen 13, 14, 24, 28 and 32. Known PEGen genes included PEGen 7 (human papilloma virus-16 early region  
25 1 binding protein; HPV16 E1BP), PEGen 8 (phosphofructokinase kinase C; PFK-C), PEGen 21 (a fibroblast growth factor-4 inducible gene; FIN 14), PEGen 26 (poly ADP-ribose polymerase) and PEGen 30 (rat espl  
homology). In the case of the PSGen cDNAs, six of six  
(100%) were novel, including PSGen 12, 13, 26, 27, 28 and  
29 (Table 3).

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PEGen 7 is expressed at ~ 4-fold higher levels in E11-NMT than in E11 cells. PEGen 7 is ~98% homologous to 16E1-BP, a cDNA encoding a protein identified using the yeast two-hybrid assay that interacts with human papillomavirus type 16 E1 protein (35). 16E1-BP encodes a 432aa protein of unknown function but does contain an ATPase signature motif (Gly-X4-Gly consensus ATP binding motif at aa 179 through 186). 16E1-BP appears to be a form of TRIP13, a protein previously shown to bind thyroid hormone receptor in yeast two-hybrid assays. The role of PEGen 7/16E1-BP in the progression phenotype in the E11/E11-NMT progression model is not known. Additional studies are necessary to determine if this gene change is associative or causative of transformation progression.

PEGen 8 is expressed at ~3- to 4- fold higher levels in E11-NMT than in E11 cells. PEGen 8 shows 100% homology to rat phosphofructokinase C (PFK-C) (36). PFK catalyzes the rate-limiting and committed step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-biphosphate. Three subunit isozymes of PFK have been identified, that form homo- and heterotetramers with differing catalytic and allosteric properties. PFK-M is specific for cardiac and skeletal muscle, PFK-L is expressed in many tissues but is most abundant in the liver and PFK-C is expressed in several brain regions and the anterior pituitary but not in liver, skeletal muscle, or several other human tissues. The cDNA of PFK-C isolated from a rat hypothalamic cDNA library is 2643 bp and encodes a protein of 765aa (-36). In a recent study Sanchez-Martinez and Aragon (37), demonstrated that PFK-C is the predominant form of PFK in ascites tumor cells (obtained from a transplantable mouse carcinoma of mammary origin), whereas PFK-L is most abundant in the normal mammary gland. These results suggest the interesting possibility that PFK-C might contribute to the malignant nature of specific target cells. The role

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presently reported of PEGen 8/PFK-C in progression in the E11/E11-NMT model remains to be determined.

5 PEGen 21 is expressed at ~3- to 4-fold higher levels in  
E11-NMT than in E11 cells. PEGen 21 displays ~98%  
homology with the fibroblast growth factor-4 inducible  
gene FIN-14 (38). FIN-14 is a novel cDNA of unknown  
function that hybridizes with a 4.5 kb mRNA that is  
10 induced 4-fold in NIH-3T3 mouse cells following treatment  
with FGF-4. The induction of FIN-14 occurs late (18 hr)  
after treatment with FGF-4 and does not occur when cells  
are treated for 18 hr with FGF-4 in the presence of  
cycloheximide (38). These results confirm that FIN-14  
15 encodes a late-inducible gene. Moreover, nuclear run-on  
assays document that FIN-14 is transcriptionally  
activated in NIH 3T3 cells following growth factor  
stimulation. Tissue distribution studies indicate  
expression of a single mRNA species in the kidney with  
low levels of expression observed in several other  
20 tissues including testis and thymus. Mouse embryogenesis  
studies indicate that FIN-14 expression occurs  
constitutively in mouse embryos between day 10.5 and  
15.5. Unlike NIH 3T3, FIN-14 was constitutively expressed  
in PC12 cells and its level did not vary appreciably in  
25 response to growth factor stimulation. The role of PEGen  
21/FIN-14 in progression in E11/E11-NMT model system is  
not currently known.

30 PEGen 26 is expressed at ~3- to 4-fold higher levels in  
E11-NMT than in E11 cells. This cDNA is identical to rat  
poly(ADP-ribose) polymerase (PARP) (39). PARP contributes  
to the ability of eukaryotic cells to contend with both  
environmental and endogenous genotoxic agents (40). PARP  
is a nuclear enzyme that binds to DNA breaks and then  
35 catalyzes the covalent modification of acceptor proteins  
with poly(ADP-ribose) (39,40). PARP activity contributes  
to the recovery of proliferating cells from DNA damage



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and to the maintenance of genomic stability, which may be regulated by effects on chromatin structure, DNA base-excision repair and cell cycle regulation (39,40). The role of PEGen 26/PARP in mediating the progression phenotype is not currently known. However, since cancer is a progressive disease characterized by the accumulation of genetic alterations in the evolving tumor (6), it is tempting to speculate that overexpression of PEGen 26/PARP in E11-NMT may facilitate the ability of these aggressive cancer cells to maintain genomic stability during cancer progression. In this context, PEGen 26/PARP may be an integral component of progression. This hypothesis is readily testable. PEGen 30 is expressed at 2- to 3-fold higher levels in E11-NMT than in E11 cells. This cDNA displays ~98.5% homology to rat *espl* (41). Rat *espl* encodes a 24-kDa nuclear protein which is the rat homologue of *Drosophila* Enhancer of split., a gene involved in ventral ectodermal development in *Drosophila* (41). PEGen 30 appears to be a homologue of *espl*, since the message detected in E11 and E11-NMT cells (~4 kb) is larger in size than the reported *espl* transcript (1.3 kb) (41). The role of PEGen 30/*espl* in tumor progression in E11/E11-NMT model system remains to be determined.

The PSGen cDNAs, 12, 13, 26, 27, 28 and 29, consist of sequences without homology to those in various DNA data bases. Expression of PSGen 12 and PSGen 13 cDNAs is ~3- to 4-fold higher in E11 versus E11-NMT cells (Fig. 29). It is not currently known whether these genes simply correlate with or functionally regulate the progression phenotype. The identification of full-length cDNAs for PSGen-12 and PSGen-13, as well as the other novel PSGen and PEGen cDNAs, are in progress and once isolated experiments can be conducted to directly define the role of these progression-related genes in cancer progression.

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Presently demonstrated is a modified gene-identification and gene-cloning technique, RSDD, that can efficiently identify differentially expressed cDNAs. As predicted, subtractive hybridization prior to differential RNA display greatly reduces band complexity, a problem encountered in standard DDRT-PCR in which RNA samples are directly analyzed without subtraction. Unlike a previous report using subtracted cDNAs processed through successive rounds of PCR (25,42), common bands were eliminated using reciprocally subtracted cDNA libraries that had not been processed using PCR. In addition to subtraction hybridization, the discovery of differentially expressed genes was further streamlined by using reverse Northern analyses with isolated cDNAs. With 3 single anchored oligo dT primers and 18 arbitrary 5' primers, 72 bands were identified that displayed differential expression using reverse Northern analysis. Currently, 38 cDNA species have been analyzed by Northern blotting and 31 (~82%) displayed differential expression in E11 versus E11-NMT cells. Sequence analysis of the cloned cDNA fragments revealed 16 different genes, including 11 novel genes not reported in recent DNA databases. RSDD represents a method of choice either as a more efficient and less time consuming modification of the differential RNA display strategy or as a screening methodology for identifying differentially expressed genes in reciprocally subtracted cDNA libraries. Moreover, the ability of RSDD to identify differentially expressed genes that are dissimilar to those recognized using standard DDRT-PCR or subtraction hybridization indicates that this approach will be a valuable adjunct in cloning the complete repertoire of differentially expressed gene changes occurring between complex genomes.

References For Second Series of Experiments

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**What is claimed is:**

1. A method for identifying differentially expressed nucleic acids between two samples, comprising:
  - 5 a. selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids;
  - b. performing reciprocal subtraction between the nucleic acid samples to produce two  
10 subtracted nucleic acid samples;
  - c. amplifying the two subtracted nucleic acid samples; and
  - d. comparing the two subtracted nucleic acid  
15 samples to identify differentially expressed nucleic acids.
2. A method for identifying differentially expressed nucleic acids between two samples, comprising:
  - 20 a. selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids;
  - b. amplifying the two nucleic acid samples;
  - c. performing reciprocal subtraction between  
25 the amplified nucleic acid samples to produce two subtracted nucleic acid samples; and
  - d. comparing the two subtracted nucleic acid  
30 samples to identify differentially expressed nucleic acids.
3. The method of claim 2, wherein the two subtracted nucleic acid samples from step c are amplified prior to the comparing of step d.
- 35 4. The method of claim 1 or 2, wherein the each of the nucleic acid samples comprises a library of nucleic acids.



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5. The method of claim 1 or 2, wherein the nucleic acid samples are mRNA or cDNA derived from mRNA.
- 5 6. The method of claim 1 or 2, wherein the nucleic acid samples are obtained from total RNA from E11 and E11-NMT cells.
- 10 7. The method of claim 1 or 2, wherein the first and second nucleic acid samples are obtained from cells that differ in their exposure to external factors or in their gene expression.
- 15 8. The method of claim 1 or 2, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.
9. The method of claim 1 or 2, wherein the amplifying of step (d) comprises PCR amplification.
- 20 10. The method of claim 9, wherein the PCR amplification uses a set of random primers.
- 25 11. The method of claim 9, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer.
12. The method of claim 9, wherein the 5' primer is an arbitrary primer.
- 30 13. The method of claim 1 or 2, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the libraries.
- 35 14. The method of claim 1 or 2, further comprising PCR amplifying the first and second nucleic acid samples.

15. The method of claim 1 or 2, further comprising reamplifying differentially expressed nucleic acids.
- 5 16. The method of claim 1 or 2, wherein the comparing of step (e) comprises comparing the quantities of the two amplified differentially expressed nucleic acids.
- 10 17. The method of claim 1 or 2, wherein differences in the quantities of nucleic acid between the two subtracted libraries are electronically quantified.
- 15 18. The method of claim 1 or 2, wherein the libraries of step (b) are constructed with  $\lambda$ -ZAP cDNA library kits.
- 20 19. The isolated nucleic acid identified by the method of claim 1 or 2, wherein the nucleic acid was not previously known.
- 20 20. The isolated nucleic acid of claim 19, wherein the isolated nucleic acid is the nucleic acid designated PSGen 12.
- 25 21. The isolated nucleic acid of claim 19, wherein the isolated nucleic acid is the nucleic acid designated PSGen 13.
- 30 22. The isolated nucleic acid of claim 19, wherein the isolated nucleic acid is the nucleic acid designated PSGen 23.
- 35 23. The isolated nucleic acid of claim 19, wherein the isolated nucleic acid is the nucleic acid designated PSGen 24.
24. The isolated nucleic acid of claim 19, wherein the

isolated nucleic acid is the nucleic acid designated  
PSGen 25.

- 5           25.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
            PSGen 26.
- 10           26.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
            PSGen 27.
27.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
            PSGen 28.
- 15           28.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
            PSGen 29.
- 20           29.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
            PEGen 13.
30.   The isolated nucleic acid of claim 19, wherein the  
25           isolated nucleic acid is the nucleic acid designated  
            PEGen 14.
31.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
30           PEGen 15.
32.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated  
            PEGen 24.
- 35           33.   The isolated nucleic acid of claim 19, wherein the  
            isolated nucleic acid is the nucleic acid designated

PEGen 28.

5           34.   The isolated nucleic acid of claim 19, wherein the  
isolated nucleic acid is the nucleic acid designated  
PEGen 32.

10           35.   The isolated nucleic acid of claim 19, wherein the  
isolated nucleic acid is the nucleic acid designated  
PEGen 42.

            36.   The isolated nucleic acid of claim 19, wherein the  
isolated nucleic acid is the nucleic acid designated  
PEGen 43.

15           37.   The isolated nucleic acid of claim 19, wherein the  
isolated nucleic acid is the nucleic acid designated  
PEGen 44.

20           38.   The isolated nucleic acid of claim 19, wherein the  
isolated nucleic acid is the nucleic acid designated  
PEGen 48.

            39.   The isolated nucleic acid molecule of claim 19 which  
comprises:  
25           (a) one of the nucleic acid sequences as set forth  
in Figure 35;

            (b) a sequence being degenerated to a sequence of  
(a) as a result of the genetic code;

30           (c) a sequence encoding one of the amino acid  
sequences as set forth in Figure 35.

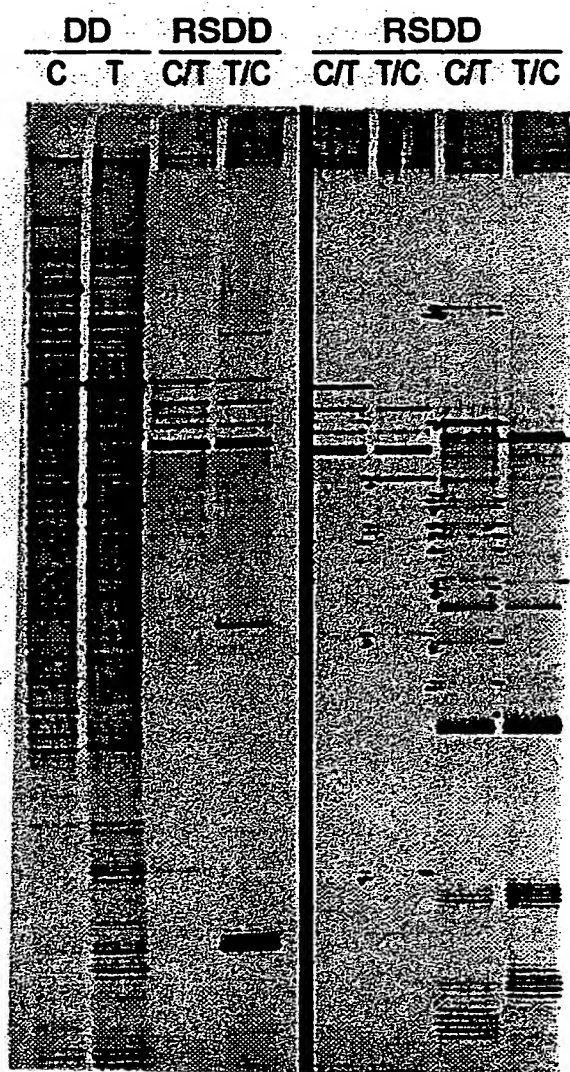
35           (d) a sequence of at least 12 nucleotides capable  
of specifically hybridizing to the sequence of  
(a), (b) or (c)

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40. A purified polypeptide comprising one of the amino acid sequence as set forth in Figure 35.

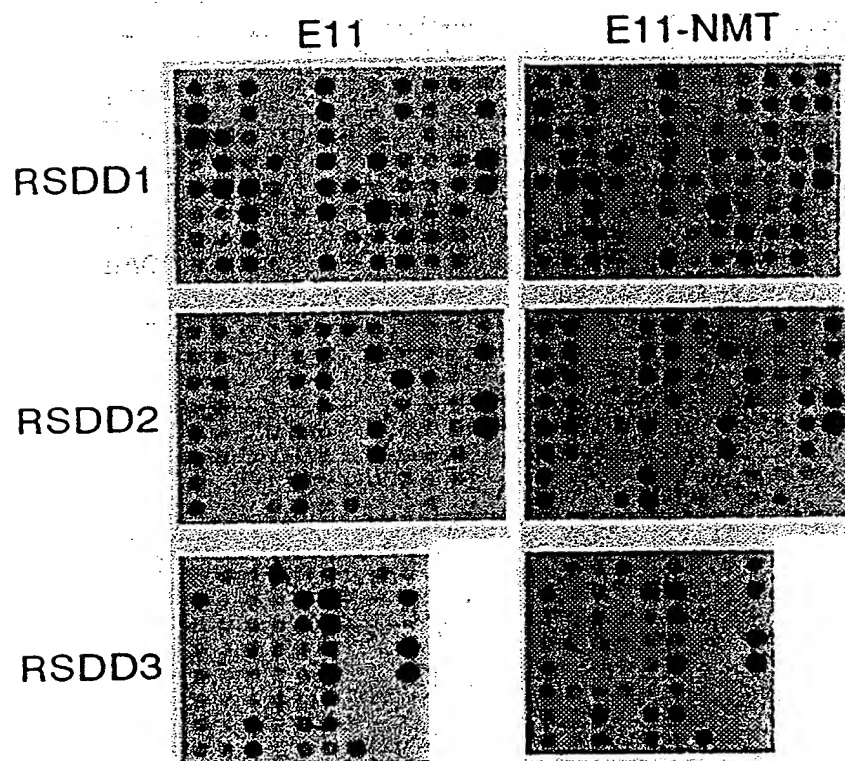
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FIG. 1



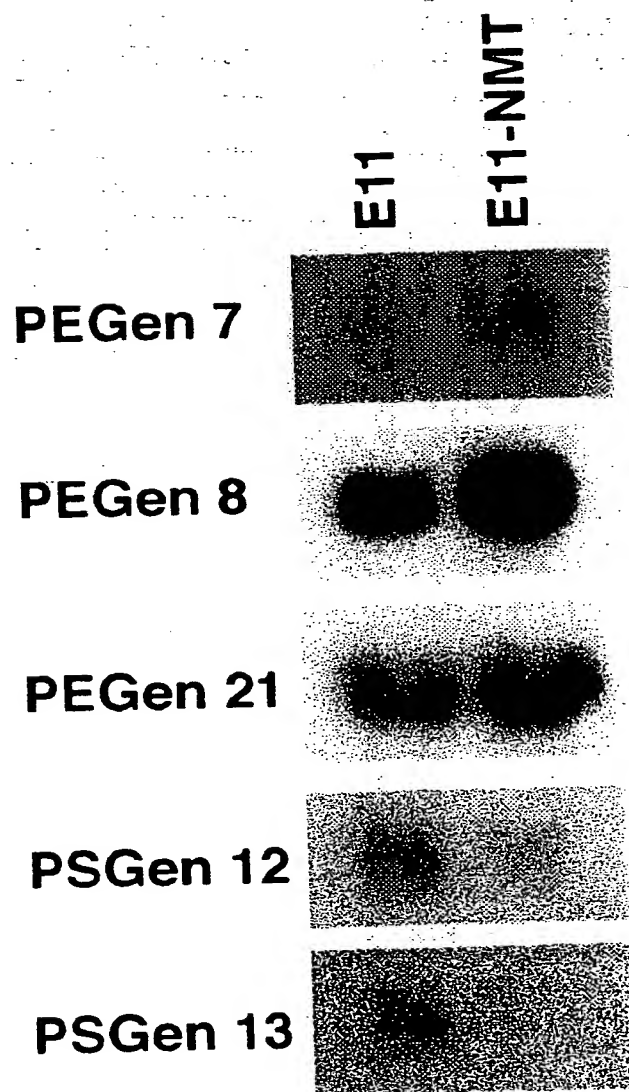
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FIG. 2



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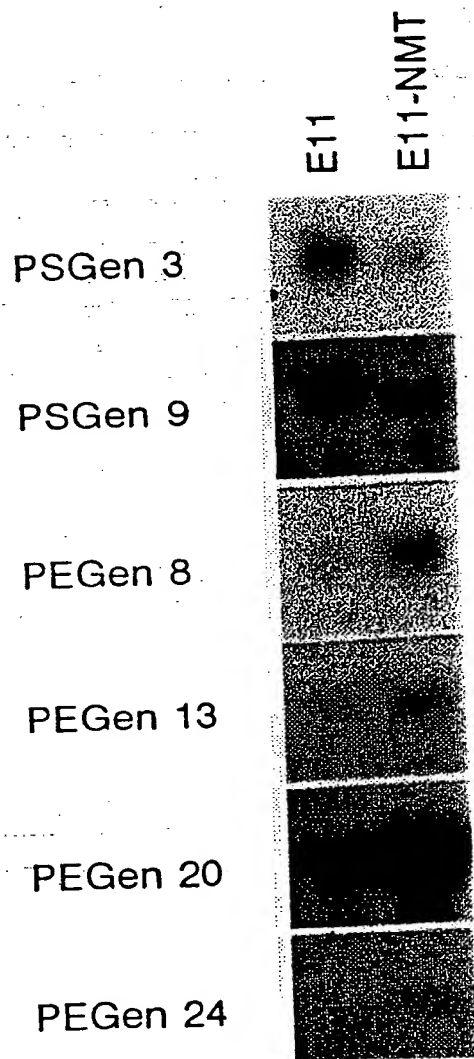
FIG. 3A





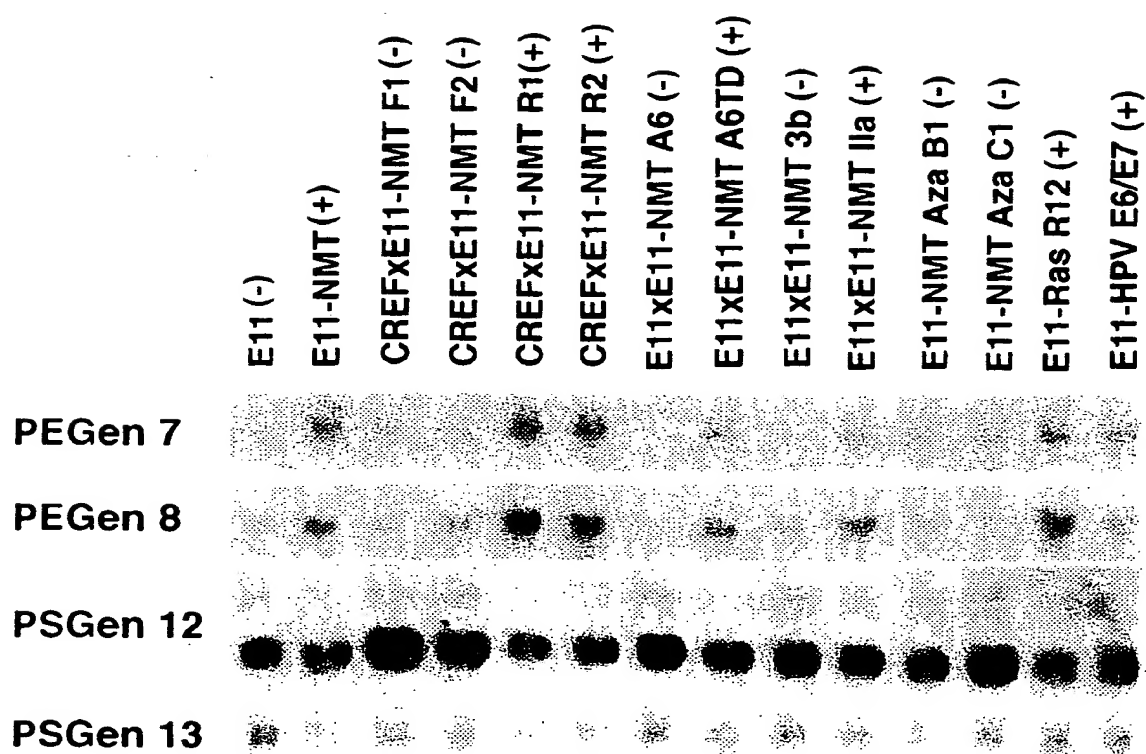
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FIG. 3B



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FIG. 4



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**FIG. 5**

PEGen 7-90% homology to human HPV16 E1BP

```

TAAANCGGTG GTACTGCTGC ACGGTCCTCC GGGTACTGGA AAGACATCCC
TTTGTAAGGC ATTAGCCCAG AACTGACCA TCAGACTGTC AANCAGGTAC
CGGTATGGCC AGTTAATTGA AATAAACAGC CACAGCCTAT TTTCTAAGTG
GTNTTCAGAA AGTGGCAAGT TGGTAACTAA GATGTTCCAG AAGATTCANG
ACTTGATTGA TGATAANNAA NCTTTGGTGT TTGTCCTGAT TGATGANGTA
AGCACTCANN GGTACTCATT CTTNGTCTGC ATTGCCTCTT GCTATTACTG
CCTGATCCCT CTCATTTGGT TCACTGTGTC GCNANCTCTT TTCTATGGAT
CTTTTCCNAN CCACCCGTTT C

```

**FIG. 6**

PEGen 8-Rat phosphofructose kinase C

```

GTGACGTAGG GTCTGTTGCG TCAATGGTTA TAGCAAGTGA TGCTCTCTGA
TTATTACTGC TGACAATACT CGGCCAACAA TTCTTGCATA GAGTGCTGAT
AAATAACTAT GTTACAAAAA GGGGTGGTCC CTGGAGAACA TTACAGGCTT
CCCTAGGTAA GTGTGCAGGT CAGGAGACGG CATATTCAAT CAGATGGCTG
ATAGTTCTCC GTGGTTATGC ACCGGCTCCA GCTTGCCTAC GTCAC

```

**FIG. 7**

PEGen 13-Novel

```

GCAGCATGAT GAATTTAATG CAACAGTCAT AGCAGGGCAA GGGGAGAGAA
AGGCAGATGG ACTATCTGCA TCATCAAGCG AGGGCTTG TG TCGGCGGCTA
TGTGCAGAGA CGAGCAGGGC GAGGCACTTA AAAGCTGCTN GATGAAAATC
CACCCAGGAG AANTCTGGGC CTACGTCA

TGACGTAGGC CCAGACTTCT CCTGGGTGGA TTTTCATCCA GCAGCTTTTA
AGTGCCTCGC CCTGCTCGTC TCTGCACATA GCCGCCGACA CAAGCCCTCG
CTTGATGATG CAGATAGTCC ATCTGCCTTT CTCTCCCCTT GCCCTGCTAT
GACTGTTGCA TTAAATTCAT CATGCTGCCA AAAAAAAAAA A

```

**FIG. 8**

PEGen 14-Novel

```

GCCATAAATA CACTTTATTT CATTCGAAAT GCATAATCAC ACTGGGAGCA
CTCCCTTTGG AGCACTCCTC TAGCAGCAGG TCCGAAGTGC TCCAGCATCG
TCAGCTGGCT CCAACACCTA CGTC

```

**FIG. 9**

PEGen 15-Novel

```

TTTTTTTTTT TTTGGAAACA GAATAAAGTG CTTTATTCTC TGGCTGGCTC
TCCTACGTCA C

```

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**FIG. 10**

PEGen 21-94% homology to mouse FIN 14

```

TCGGCGATAG CATTGGAGCA AGTCTTATCA GCAAGCAATG TTTTCAGTTA
TGTTTCAAAG TTAAGAATGG GTTTAAACTT GCTGAACGTA AAGATTGACC
CTCAAGTCAC TGTAGCTTTA GTACTTGCTT ATTGTATTAG TTTANATGCT
AGCACCGCAT GTGCTCTGCA TATTCTGGTT TTATTAAAAT AAAAAGTTGA
ACTGCAAAAA AAAAAA

```

**FIG. 11**

PEGen 24-Novel

```

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TNGCCAGGCT
ATGTCTCAGA CTTTATTATT ATTATTATTA TTATTATTAT TATAAATAAA
ACATGTNCCT TCAATTAGGT TACAANAGTA TTTATCTCCA TAACGCTTCT
TCATACATCC TTAGTTTTGG ATTAAAGTAC CATCCACCCC AACTCAAACCT
GTAACCCCCA GTAATCCCCT CTAACGTGGA AATTTCTGGT TTAACAACTC
AGTTAACTGC CCCACAAACA GTGGGAGGCC GCTCTTGCAT GGCTATGCCA
CGTAACCCTT CACTGCTTCA CTTCTTCGCT GGCT

```

**FIG. 12**

PEGen 26-Rat poly ADP-ribose polymerase.

```

GACCGCTTGT ACCATCCAAC TTGCTTTGTC TTCTGCAGAG AGGAGGCTAA
AGCCCTTGAG CTGGCTGGCA CTGTACTCAG GCCGGAAGCC CAGCTCGTCC
CGGTTCTTGA CAAAGCAAGT TGGATGGTAC AAGCGG

```

**FIG. 13**

PEGen 28-Novel

```

TGCCGAGCTG GGTATTGTGA CGGTTGATAA TGGCGGCATC ATGTTGCCAG
GTACCGGGTA AGCAGACCTC AGAGCACAGC TTATTGTCCA GTGCTTTCAC
GCTCGCGACG TCAAAGTCAT TGTTATTGTC AACTCCATG CCTAGAAATG
CGCATGTCCT CTGGCCATCT TCTTGACAG GGGATCTGTC CTCTTCCTCC
ATGATATCAT TTCCCTCTGC ATCCTGCTCT CCAGCTGGAA GGCCAGCAAA
ATTGCTGTCT GGGGACTCTG CTGGGGTCTC CTCTCTTCT GAAGGGGCCC
TGCTAGCAGC TCGGCA

```

**FIG. 14**

PEGen 42-Novel

```

AGGGGTCTTG ATGGAATTGG GTCGGACATC TTAGTGACCT GTGAATTCTT
CTGTGGAGGC TGAGTCTCAC GTAGCCGAGT TTAATATCTG TGCTATTTAC
TAAAGTATCT GCCACCAAAT TGTACCAACT CATAGTTTTA TATGAATGTT
GATGAGTCTG TATCATAAAT AGAATTGTTG ATACATCCTT AATTTGTGCA
ATATTGTATG AAGAAGATTG TTATCAATTA AAACCACGCC TCTTTATGAT
CCTNNNAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
AACCNCCTCA AATCCATNGG TTCTAACCCA AAACCCT

```

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**FIG. 15**

PEGen 43-Novel

```

TTTTTTTTTTT CATAACCAT CAAACCAATT TTATTTCTAT AGCAACGTTT
CTCACGTCTG AACCTGAGAA TAAGTCACCA GCTCTTGACA GTAAACATGG
GCCCTATCAA ATTATATTAG ACTCCTCAGT GTCCCGCCAT GTGGCCTTGC
ACCAAATCAA TTAGTTTGAG GGCCAAAATC CTGTTGGGTT TCAAATAAAG
TGTCAGGTCA TAAGGAGGGG GAGGGACTCA ATTCATGGGA ACATTTTAC
CTGTTCAAAT AGATAAACTG AATTGCCCTA TCTGTGGTCA CCTGGATCCA
AGACCTT

```

**FIG. 16**

PEGen 44-Novel

```

CCCTGACGAT AAATGGTAAG GAACTTTTTT TTTTTTTTTT TTTTTTTTTT
TTTTTTTTTNC GAAATAAACA AACACAGCTT ATTATTTGGG GGAACATTAA
NTTCTATAAN TGAACACAAA ANAAAATTAA NANTTAATGG GGGGGTANAA
GGGACTTTGA ATCTATCTGG TATCATGACA TTGAAGCANA NACCTGANTG
ACCAGAAAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGGTTTC
ATATGAGCTA GTGTTACAGG CTTTATTAGT CTATTAGTCA GGGACC

```

**FIG. 17**

PEGen 48-Novel

```

AATCGGGCTG GATGGGTGTA TCCGGCACTG TTTCGTAGCG GCAGCAACTG
GGTGCTTCTA TCTGAAAGCG GGCTTCACAA AAACACTGCG GCCACCCGAC
TCGCTGCGGC ATCGCCCGGT GGCGAGTACC GTATCGCCTT TCCTGGTGCA
GAAGAAGTGT TTACAGGAGG CGGTCATTTA CCGCAATCTG ATTCTGTTTT
TTATTCTCCC TGGCGGGTGA TCGCGATCGG CAGTTTGAAA ACGATCGTTG
AATCCACGCT CGGGAATGAT GTGGCTTCGC CGCCAACGCT TACTGACATT
TCATTTGTAC AGCCCGATT

```

**FIG. 18**PSGen 1-80% homology to *B. taurus* supervillin

```

GCCGAGCTGT GTAAAACCAT CTATCCTCTG GCAGATCTAC TTGCCAGGCC
ACTCCCAGGG GGGGTAGACC CTCTAAAGCT TGAGATTTAT CTTACAGATG
AAGACTTCGA GTTTGCACTC GACATGACCA GAGATGAATT CAACGCACTG
CCCACCTGGA AGCAAATGAA CCTGAAGAAA GCGAAAGGCC TGTTCTGAGG
GTGAGATGAC AGCCACAGAG AGGTCACCTG CACTAGACCA GAAAGTGGAT
GGAGATATAT ATTTGGACTG GTGTTTTTTT CTGTCAG

```

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**FIG. 19**

PSGen 2-91% homology to human HTLV-1 Tax interacting protein

ATCGGGCTGC	AGATTGGAGA	CAAGATCATG	CAGGTGAACG	GCTGGGACAT
GACCATGGTC	ACTCATGACC	AGGCTCGGAA	GCGGCTCACC	AAACGTTCGG
AGGAAGTGGT	CCGCCTGCTG	GTGACTCGGC	AGTCTCTGCA	GAAGGCCGTA
CAGCAGTCCA	TGCTGTCATA	GCTGTAGTCA	GCCTAGACTT	CTGCCCCACTG
ACCTTTTNGG	GCACTGAGAA	CACATCCACG	CTCTGTCTGT	ATCTAGTTCT
GGCTTCTGCT	GTGTGCTANG	CCCCAGCTCT	GAGGAGTAAC	AGCTGATCCC
AAAGGTCCAA	GCCAACCTTC	TTACCCCTCA	GCCCCCANCC	CGAT

**FIG. 20**

PSGen 4-Rat proteasome activator

TTTTTTTTTTT	TTTGGGCAAC	TATGTATTTA	TTGTGTTTGG	AAGGCAGAGT
GAGGGAGGAG	ACCCAGCAG	GAAGAAGACT	GGGTGCAGTC	TAGAGTTCCT
AGTCAAGAGT	AGGAAGGTTT	CTGTTATACC	CATCATAGAA	CGAGAGAGGG
GGCTCAATAG	ATCATCCCCT	TTGTCTCTCC	ACGGGGCTTC	TTGAGCTTCT
CAAAGTTCTT	CAGGATGATG	TCATATAACA	CAGCATAAGC	GTTACGGATC
TCCATGACCA	TCAGCCGGAT	CTCCTGGTAT	TCCGCCTCGT	CCAGCTCGGC

**FIG. 21**

PSGen 10-Rat Ferritin Heavy Chain

AANATCTGCT	TAAAAGTTCT	TTAATTTGTA	CCATTTCTTC	AAATAAAGAA
TTTTGGTACA	AATTAAAGAA	CTTTTAAGCA	GATGTTTTGG	TGCAACTAAT
AGAAAAGATA	AAGGCAGCCT	GACATGCATG	CACTGCCTCA	GTGACCAGTA
AAGTCACATG	NCCTTGGGAC	GTCAGCTTAG	NTTTATCACN	GTGTCCCAGG
GGTGCTTGTC	AAAGAGATAT	TCTGCCATGC	CAGATTCAGG	GGCTCCCATC
TTGCGTAAGT	TGGTCACGTG	GTCACCCAGT	TCTTTAATGG	ATTTACCTG
CTCATTCAGG	TAATGCGTCT	CAATGAAGTC	ACATAAGTGG	GGATCATTCT
TGTCAGTAGC	CAGTTTGTGA	AGTTCCAGTA	GTGACTGATT	CACACTCTTT
TCCAAGTGCA	GTGCACACTC	CATTGCATTC	AGCCCGCTCT	CCCAGTCATC
ACGGTCACNT	A			

**FIG. 22**

PSGen 12-Novel

TGACGTAGGG	CCGAGAGCAA	CAAGCACAGA	ACTCCTTCTC	CAGTTTCACC
CTGATGAAGT	TGAGGCACTC	TTCTGCACTG	GGAGGGGCCA	GCCTGGGGGC
CAGGCACATT	GGACACCACC	TTCCCATGGA	CTACAGCGTC	AATGCCATTG
CCTTCTATTTC	CTATACCTTC	TAGGGGCTGC	CCCTCTTCCC	ATTCAGCCAA
CACTGAGTGT	TGGGAGATTT	CTCTTTTTTA	AAAACACATG	AGAAAATAAA
TGCACTTTAC	TCCCTCCCCA	AAAAAAAAAA		

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**FIG. 23**

PSGen 13-Novel

GTAGGCAATA	AAATGTTTTTC	AGAGGTGCGA	AAAAGCTTTT	GTTTTCTTAA
ACCATTCTTA	GTCTCTGCCA	CACTTGACAC	TCCGTCAAAG	TGAGAAGCGA
ACTAAAGACC	AACTGCGGTG	GAAAATATTA	TGTTTATGTA	ATAAAAAAAAA
ATCATGTAAC	TGCAAAAAAAAA	AAAAAAA		

**FIG. 24**

PSGen 23-Novel

TGCCGAGCTG	AAAACATACA	TCCGCACCGG	GTTGAGATAG	CTGGCCCTCC
GTCCCCGGGC	ATACTCTTTG	GATAAGAACC	CCGGCCTTGT	TACCAGGTAC
CGGAGTGAGC	TGAAAAATTT	ACCGTCGAAA	TGGGTGATGT	CCTGGAAAAA
ATGGTTCACC	AGCTGCCAGG	CAGATTCTTT	GGGTTCACA	TTTTCTGCC
CACAGATGTG	GCAGAAGCGG	TCAAGTAATG	CAGCATTACA	ATTGAGGCAG
ATCTTTTCTT	TTCTTTCCTT	GGAGTGGCTC	AACCAGCGAT	TTTGGTTAAA
AATAATCAAA	AAAGCGACGG	CAAACTTTT	GTTATATTCC	CGCCTGTGGC
ATTTGAACTG	TGCCCGGCAA	CCGAATAACT	TTTAATTTTG	AAAATAAAAT
GCATACTAGA	TTTTTAGCGG	TTGCCTCCTG	GCCATTGCTT	CAGGCGCCNG
CACAGCGTCA	GCCCAGTTTT	ACCACNANGA	ATATCCTAAG	CGTTGAAACA
GGGCACAGCC	GAAAAAAACN	CTGGCNACAA	AAAANATCCG	GACATCCTTT
TTCCAATTTT	GAAACCGAAN	GCNCGCAAAC	NAAGGTTCTT	CGGGAAAAAA
AATCGCCAAA	ATACNCGANA	TCAAACNTNC	CAA	

**FIG. 25**

PSGen 24-Novel

TGCCGAGCTG	GGGGGAGTTC	CAGGAATTTG	TGGACTATTT	CCAGGAGGAA
TTGAGGAATC	TAGAAGTAAT	AAGAACTTCA	CAAGTAGAAC	AACAGAGTTA
ATTGACCTCT	ATCCTTAAGA	GTTACCAGAG	AATTATTAAA	AAACTAAAGA
ACAATCAAAG	CCTGGTCCTG	TGCCACCACC	CAAAAACATG	TATAGCCTAT
GTGCAGCTCG	GCA			

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**FIG. 26**

PSGen 25-Novel

CTCANAGGGC	NNNTTNGNGG	NCNTCATGCN	CCAGGNTCCN	NCCCCCANAN
GANCNCCNG	GTAAACTACA	CNGGAGTACT	TAAGTGGACA	NNCCACATGC
GANGGNCAAG	GGGATCACCN	TCNCTCCTNC	AGNCTNTNCG	TGNCTCTCCT
GTNCNTNCAC	TGCCNCANAA	NGGANGCNCN	NNCTCCTATC	TGTNTACAGN
AAACNTNGCN	CTNNCTCTAA	GCTCNCCCAC	TNTGTGGAAA	GGCNATGTGT
GCGTGCCTCT	CCCCTATCAC	GGCNGTTTGC	NAAANGGGGA	TGTNCTGCNC
GGCGATGAAG	TTNGGTCACT	CCATGTTTCC	CAGTCCNACC	TGTTAGACNA
AGNATTGNAN	TGTGATACGA	CTCNCTGTAA	GGGGANTNGC	GGACCCAGTA
TGTTTGGCCC	NACNNCCACT	TCTTTAAATG	GTGGCTAACG	GCGCTTCCTA
GNATAAACAC	TATTGGTCCC	CCCCTCTGCA	GNACCCNTTA	CTTCCGNANA
AAAATTGTTG	TCNTGATCCG	CGACAACCAC	ACCGTCTGTN	GNTTTTAGTT
GCAACNCNNA	TCNCTCCAAA	AAAGTTTCAG	AAATCTTCAT	TTTCCCNGGT
TGAGCCCNTG	ACAAACCCCT	NAGGATTTGT	CGAATGTAAA	GTCTCCNGAT
CTTCAATAAA	NNTCCAAAAG	NCTANCGAT		

**FIG. 27**

PSGen 26-Novel

TCACTGGGCN	NNNTGGTNGN	CGTCATGCNN	NAGGTTCCNN	CCCCCANNANG
AACCTCCNGG	TAATCTACAC	NGGAGTCTTA	AGTNGACAAN	CCCACACTGC
GANGGTCAAG	NGGATCACCA	TCNCCNCCTC	CCAAGCTTNT	NCATTGATGC
TCTCTCTGTT	CCGTNCCCTG	CCGCTACACA	TGGANGCTCT	TNCTCCTTNT
CTCNTCTTAC	NANNCAAACA	TTGCCCTNTC	TCATA	

**FIG. 28**

PSGen 27-Novel

GGGAANGGGA	NNAAAAAGGA	ATTTTTTNGG	GGGGGGNTTN	TCTGGGAAAN
TTTTTTTTTT	TTTTTGGNAA	AAANGGGGGG	GGAAANAANC	CGNTTTTCCC
NAAACNNGG	GGGAACNNGC	CGGGGGGGGA	AAAAAAAGGG	TTACNAAGGG
AAACCTTTNA	AANNGGAANG	GNTTTGCNNC	CCTNTNGAAA	NNTTTGCCCC
CCNNNAGGAA	TCCCNNGGNA	AACCCAANNC	CNNCNCNCNG	GGGGNCNNTN
CNANGGGACC	CCAACNCGGG	CCCNAACTNG	GGGNAAANAN	GGGCAAAACN
GGTNCCCGGG	GNAAAANGGT	ANCCCCCTC		



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**FIG. 29****PSGen 28-Novel**

TGCCGAGCTG GGGGTGAAGC ACCGGAAAAC AACCGATCCA TCTCTTATCA  
 CAGGGTCTCC AAGATCCCAA ACCCAAAGC CACATTGTTA ATTAGCCTTT  
 TTATTGTGTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT  
 TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTGGCAGC TCGGCA

**FIG. 30****PSGen 29-Novel**

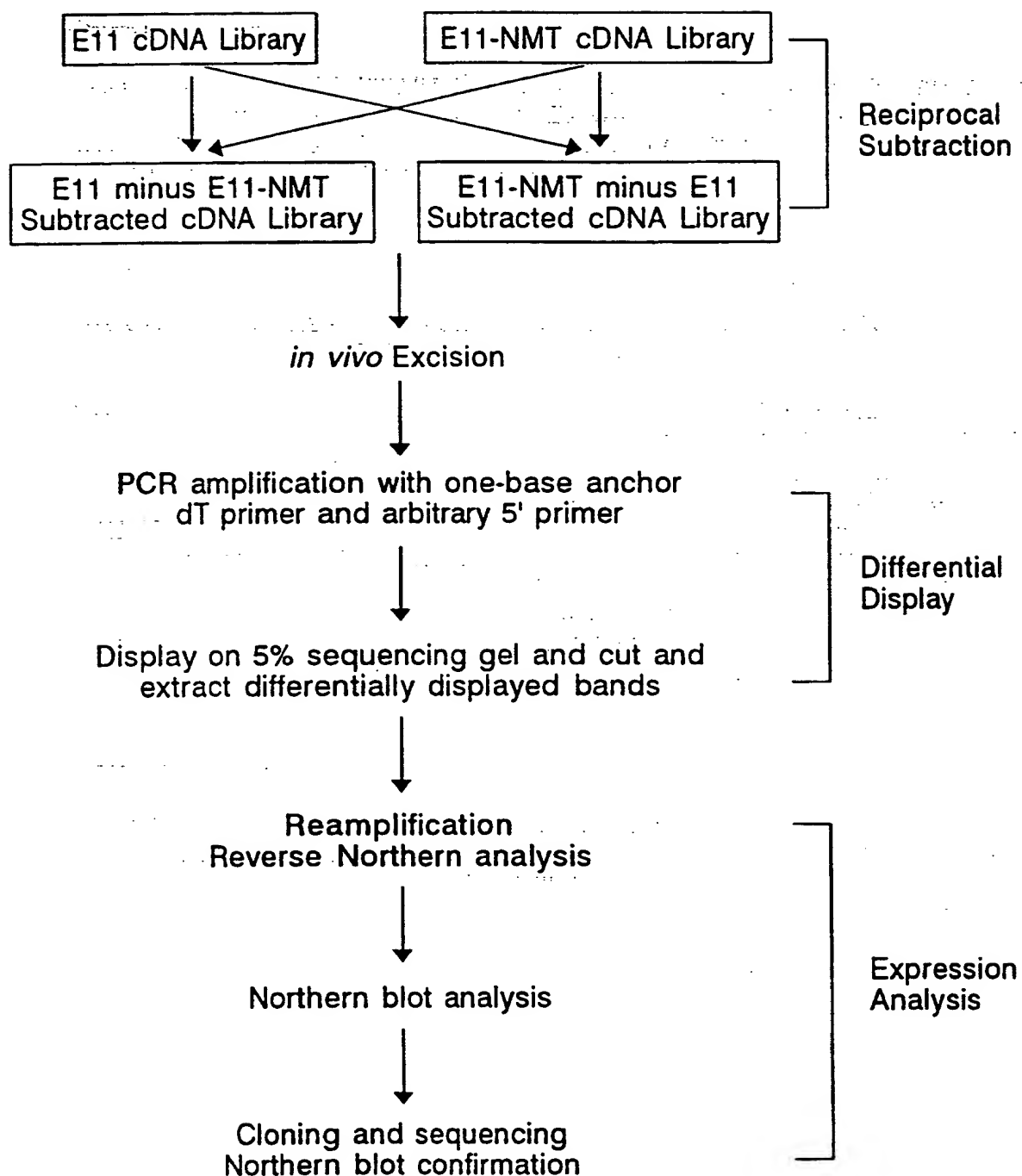
TACGGGCGCT GATTTTTACG AACATTACCT GGCAGGGAAA TTTGATAAGT  
 ATCCACTGTG GGTGGCGCAC TACCTGGTAA AAGACAAACC CCGTGTGAAA  
 AGGCCCTGGA CTTTTTGGCA ACACAACGAA ACCGGCCACG TGAATGGCAT  
 CCGGTCTTAT GTGGACTTCA ATGTTTTCAA CGGGGACAGC ACAGATTTTG  
 CCGAACTATT AATGAAATAA TGCAGAATTT CGCTTTTCAA ATAAGCCCAT  
 GGATCCTGAC GTAAAATATT TCCTGCTGGT GATCGTGCAG TCCATTTCGA  
 TGCTCATACT TTGGCTGATG CTCAACATGA CCTTTGGGAT CTATTTTAAT  
 TTTGCTTTCC CCGACAATGG TTTGACGCTT GGCAACATCA TTTATTACCT  
 CTTCTGCTG GGCAGCTCGG CA

**FIG. 31****PEGen 32-Novel**

TNCATANGCC CTGAGGTGGG GACGAAGCCC GAGTCCGTCC TGACATGTTT  
 CCAGTGGAAA AGATTTTGTT NTGAGCGTTN CTTTCTNNTT TTTTTNNNT  
 TGNTTGTTNN ATGTTTTTGT TGTGTGTTTN TTNAACTGT NTGTTGNCAN  
 TTCAACATNA ANGGNAGGNA ANTNTGTGNC TNCNTTGCAN TGTNNCATGN  
 TNCCCANANC CCAAAAAAAA AAAAAAAA AAAAAGAGTA CAAATATCAC  
 AAAATTTGAC ATTTTTGTAA TAATACTTTG GTTGTTGTTT GGTGACGGCG  
 ATTG

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FIG. 32



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FIG. 33A

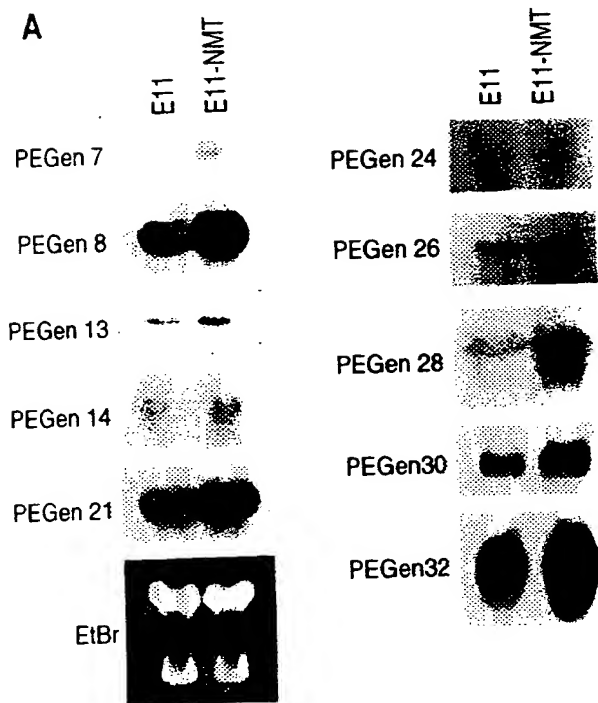
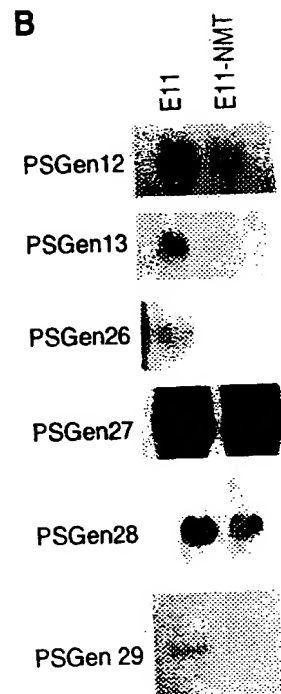


FIG. 33B



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FIG. 34A A

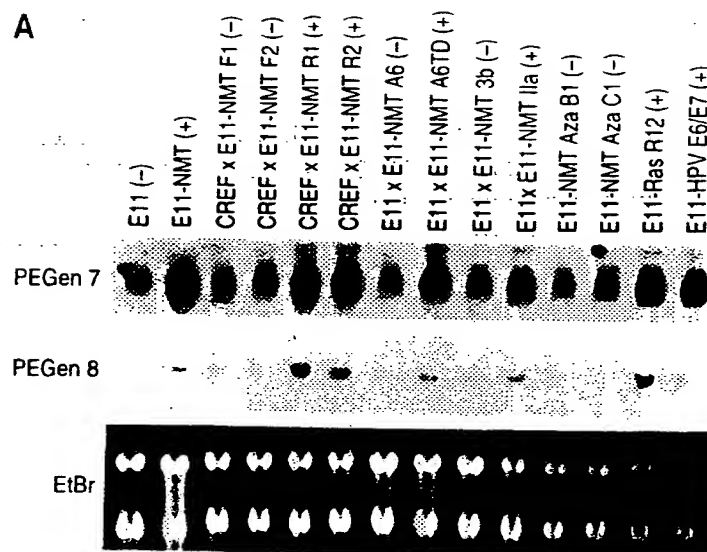
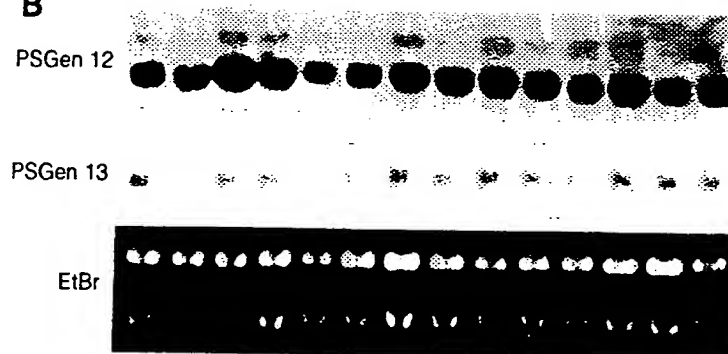


FIG. 34B B



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**FIG. 35A****PSGen 12 cDNA Sequence**

```

GCGGTGGTGA CGGTAGTATG GCCGCACTTT ATGGTGGCGT GGAAGGGGGA
GGCACACGGT CCAAAGTCCT TTTACTTTCT GAGGATGGGC AGATCCTGGC
AGAAGCAGAT GGACTGAGCA CAAATCACTG GCTGATTGGC ACAGGTACCT
GTGTGGAGAG GATCAATGAG ATGGTGGACA GGGCTAAACG GAAGGCTGGA
GTGGATCCTC TGGTACCCCT TCGAAGCCTG GGCTTGTCCC TGAGTGGTGG
GGAGCAGGAG GATGCAGTGA GGCTCCTGAT GGAGGAGTTG AGGGACCGAT
TTCCCTACCT GAGTGAAAGT TACTTCATCA CCACTGATGC AGCAGGTTCC
ATCGCCACAG CTACACCGGA TGGTGGGATT GTGCTCATCT CTGGAACAGG
CTCCAAGTGT AGGCTTATCA ACCCTGATGG CTCTGAGAGT GGCTGTGGTG
GCTGGGGCCA CATGATGGGA GACGAGGGAT CAGCCTACTG GATTGCACAC
CAAGCTGTGA AAATTGTGTT TGA CTCCATT GACAACCTGG AAGCAGCTCC
TCATGATATT GGCCATGTCA AGCAGGCCAT GTTCAACTAC TTCCAGGTGC
CAGATCGGCT AGGAATCCTC ACTCACTTGT ATAGGGACTT TGATAAGTCC
AAGTTTGCTG GATTTTGTCA GAAAATTGCA GAAGGTGCAC AGCAGGGAGA
CCCTCTTTCC AGGTTTCATCT TCAGAAAGGC TGGGGAGATG CTGGGCAGAC
ACGTTGTGGC AGTATTGCCA GAGATTGACC CAGTTTTGTT CCAAGGGGAG
CTTGGCCTCC CCATTCTGTG TGTGGGCTCA GTGTGGAAGA GCTGGGAGCT
ACTGAAGGAA GGCTTTCTCC TGGCACTGAC GCAGGGCCGA GAGCAACAGG
CACAGAACTC CTTCTCCAGT TTCACCCTGA TGAAGTTGAG GCACTCTTCT
GCACTGGGAG GGGCCAGCCT GGGGGCCAGG CACATTGGAC ACCACCTTCC
CATGGACTAC AGCGTCAATG CCATTGCCTT CTATTCTAT ACCTTCTAGG
GGCTGCCCCT CTTCCCATTC AGCCAACACT GAGTGTTGGG AGATTCTCT
TTTTTAAAAA CACATGAGAA AATAAATGCA CTTTACTCCC TCCCCAAAAA
AAAAAAAAAA AAAAAAAAAA AAAA

```

**PSGen 12 Protein Sequence**

```

GGDGSMALY GGVEGGGTRS KVL LLS EDGQ ILAEADGLST NHWLIGTGTC
VERINEMVDR AKRKAGVDPL VPLRSLGLSL SGGEQEDAVR LLMEELRDRF
PYLSESYFIT TDAAGSIATA TPDGGIVLIS GTGSNCRLIN PDGSESGCGG
WGHMMGDEGS AYWIAHQAVK IVFDSIDNLE AAPHDIGHVK QAMFNYFQVP
DRLGILTHLY RFDKSKFAG FCQKIAEGAQ QGDPLSRFIF RKAGEMLGRH
VVAVLPEIDP VLFQELGLP ILCVGSVWKS WELLKEGFLF ALTQGREQQA
QNSFSSFTLM KLRHSSALGG ASLGARHIGH HLPMDYSVNA IAFYSYTF

```

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**FIG. 35B****PSGen 13 cDNA Sequence**

```
GGCACGAGCT CTCCTCGTCC CCTCCCTTCT CCACTGCAGC CTTTCTCTTA
GCCCCGAACCA CTTCCTTCTT CTGCTTGTTT CTCCCTAGGG CGCGGAAGCT
GAGTGCAGGG TTCAGACCCA CGCGGCGAGC AGCTCTTCAG TGAAGAAGGA
AGCAATCGGA GGGTCAGCAA TGAACGTGGA GCATGAGGTT AACCTCCTGG
TGGAGGAAAT TCATCGTCTG GGTTCACAAA ATGCCGATGG GAAACTGAGT
GTGAAGTTTG GGGTCCTCTT CCAAGACGAC AGATGTGCCA ATCTCTTTGA
AACCGTTGGT GGGAACTCTG AAAGCCCGCA AAACGAAGGA AGATTGTTAC
GTACGCAGAA GAGCTGCTTT TGCAAGGTGT TCATGATGAT GTTGACATTG
TATTGCTGCA AGATTAATGT GGTTCGCAGA TCTGGGGGTA TCTGGTAAAC
TGGAATAATT AAGTTAAAGG ACAAACATGA AGTTCCTTAT GTATTTTTAT
AGACCTTTGT AAACAAAAGG GGAAGTGTG AGAAGTCCTG TTTTATATAC
TTGGAGCAAA ACATTACAAT GTAAAAATAA ACAAACCTG TTATTTTTTT
TTTCTTAAGA AGGTAATCGG GAGACGTAGG CAATAAAATG TTTTCAGAGG
TGCGAAAAAG CTTTGTGTTT CTTAAACCAT TCTTAGTCTC TGCCACACTT
GACACTCCGT CAAAGTGAGA AGCGAACTAA AGACCAACTG CGGTGGAAAA
TATTATGTTT ATGTAATAAA AAAAAATCAT GTAAAAAAA AAAAAAAAAA
```

**PSGen 13 Protein Sequence**

```
MNVEHEVNLL VEEIHRLGSK NADGKLSVKF GVLFQDDRCA NLFETVGGNS
ESPQNEGRLL RTQKSCFCKV FMMMLTLYCC KINVVCRSGG IW.
```

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**FIG. 35C****PEGen 28 cDNA Sequence**

```

GTGTGGTGTG TCTCTCAGAC GTCCGTGACA CTTTGATCCT GCCCTGCCGG
CACCTGTGCC TCTGCAACAC CTGTGCAGAC ACCCTGCGCT ACCAGGCCAA
CAACTGCCCC ATCTGCCGGC TGCCCTTCCG GGCAGTGCTT CAGATCCGAG
CCATGAGGAA AAAATTGGGC CCTCTGTCTC CAAGCAGCTT TAACCCCATC
ATCTCTTCCC AGACTTCGGA CTCTGAGGAA CATTTCATCCT CAGAGAACAT
CCCTGCGGGC TATGAAGTGG TGTCTCTCCT GGAGGCCCTC AATGGGCCCC
TCACCTCATC CCCAGCGGTG CCTCCCCTTC ACGTTCTTGG AGATGGCCAC
CTCTCAGGAA TGCTGCCGTC CTATGGCAGT GATGGCCACC TGCCCCCTGT
TAGGACACTG TCCCCCTTG ACCACCTGTC TGATTGCAAC AGCCAAGGGC
TCAAAC TCAA CAAGTCTCTC TCCAAGTCCA TTTCCCAGAA TTCTTCTGTG
CTTCACGAAG AGGAAGATGA GCGCTCTTGC AGTGAGTCAG ACACTCAGCT
CTCTCAGAGG CTGTCAGCCC AGCATCCTGA AGAGGGACCT GATGTGACTC
CAGAGAGTGA GAACCTCACG CTGTCTCCTT CAGGGGCTGT TGACCAGTCA
TNTTGCACAG GGAATCCGCT CTCTTCCACC ATCTCCTCCC CAGAAGACCC
AGCCAGCAGC AGCCTGGCCC AGTCAGTCAT GTCCATGGCC TCCTCCCAGA
TCAGCACTGA CACCGTGTCC TCCATGTCTG GCTCCTACAT TGCACCTGGC
ACAGAAGAAG AAGGAGAGGC CCCACCTTCC CCCCAGAGCTG CTAGCAGGGC
CCCTTCAGAA GAGGAGGAGA CCCAGCAGA GTCCCCAGAC AGCAATTTTG
CTGGCCTTCC AGCTGGAGAG CAGGATGCAG AGGGAAATGA TATCATGGAG
GAAGAGGACA GATCCCCTGT GCAAGAAGAT GGCCAGAGGA CATGCGCATT
TCTAGGCATG GAGTGTGACA ATAACAATGA CTTTGACGTC GCGAGCGTGA
AAGCACTGGA CAATAAGCTG TGCTCTGAGG TCTGCTTACC CGGTACCTGG
CAACATGATG CCGCCATTAT CAACCGTCAC AATACCCAGC GCCGGCGACT
ATCACCCAGC AGCCTGGAGG ACCCTGAGGA GGACAGGCCT TCGGTATGGG
ATCCTTTGGC TGTCTGAGGG CACTGGCACC TGTACCTGGG CTTCCCCTCC
TGTCGCGCCTT CCATCTGTCC TCACTGGACC ACAGGCCTTC TGGGCATCTT
CAACAAGACA CGTGGACTTT CTA CTCTCAT GAAGGGAGGA CAGTGCAACC
CTCCACCAAC TTCATCTCCT GTAACCATGA TTCTTACCCT CTCAGAAAGT
ACCAGAAGCC TTCCTCCTGT GGGCTGATGT GTGCCAGCCA AACCAGTGG
GTCAGCTGAG CTGAGGGTCA GGGCTGGTTG TTTCTGTAGC CTTTTCTCTT
CCAAATGGAG ACCAACGAGA AANAAAAAAA AAAAAAAA

```

**PEGen 28 Protein Sequence**

```

VVCLSDVRDT LILPCRHLCL CNTCADTLRY QANNCPIRL PFRALLQIRA
MRKKLGPLSP SSFNPIISSQ TSDSEEHSSS ENIPAGYEVV SLLEALNGPL
TSSPAVPPLH VLGDGHLSGM LPSYGS DGH L PPVRTLSPLD HLSDCNSQGL
KLNKSLSKSI SQNSSVLHEE EDERSCESED TQLSQRLSAQ HPEEGPDVTP
ESENLTLSST GAVDQ SXCTG TPLSSTISSP EDPASSSLAQ SVMSSMASSQI
STD TVSSMSG SYIAPGTEEE GEAPSPRAA SRAPSEEEET PAESPDSNFA
GLPAGEQDAE GNDIMEEEDR SPVQEDGQRT CAFLGMECDN NND F DVASVK
ALDNKLCSEV CLPGTWQHDA AIINRHNTQR RRLSPSSLED PEEDRPCVWD
PLAV

```

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**FIG. 35D****PEGen 32 cDNA Sequence**

```
GGCACGAGGC GCGGCCTTCC TGCTCGCGCC CTATCGCCGC CTTCTGCTC
GCGCCCTATC GCGGCCTCCG AGTCTTCCTG CGCCCCGGGC TTCCGCCGCT
TCATTGATTT CCGTTTCTCG CCGCTGCAGC CTCCTGACAC GGTGATCCGG
GCGGGCCCCG CAGGAATTTT ATCCCCTCAC CGGCCTCACA CTAGTGTCGC
ATGTCCACTA TCCAGAACCT CCAATCTTTC GACCCCTTTG CTGATGCAAC
TAAGGGCGAC GACTTACTCC CGGCAGGGAC TGAGGACTAC ATTCATATAA
GAATCCAGCA GCGGAACGGC AGGAAGACGC TGACCACTGT GCAGGGCATT
GCGGACGATT ATGACAAAAA GAAACTTGTTG AAAGCTTTCA AAAAGAAATT
CGCCTGTAAT GGGACTGTGA TTGAACACCC TGAGTACGGA GAGGTCATTC
AGCTTCAAGG CGACCAAAGG AAGAACATTT GCCAGTTTCT TTTGGAGGTT
GGCATCGTCA AGGAGGAGCA GCTGAAGGTT CACGGATTCT AAGATGAACC
CGAACATGTG GCGAGTTTCT TAAATGGTTT TGTTGTCTAA CTCAGTTTGG
CTGCCTCGGG AGATGATTCT TTACAGTAAA CGACAGACTT TGC GTTTATT
AAATCATTCA GACTTCCACT CACGCCTGCA TGGCTACAGA AAACATGGGG
TATGTAGGCT CCTAAGTCAC AAGGAAATCG CCGTGAGGTG GGGACGAAGC
CCGAGTCCGT CCTGACATGT TTCCAGTGGA AAAGATTTTG TTCTGAGCGT
TCATTTCTAG TTTATTTTCA CTTGATTGTT AAATGTTTTT GTTGTTGTTT
TATTAAACCA TGTATGTTGC AGCTTAACAA TAAAGGAGGA AAGTCTGTGC
GTCAAAAAAA AAAAAAAAAA AA
```

**PEGen 32 Protein Sequence**

```
MSTIQNLQSF DPFADATKGD DLLPAGTEDY IHIRIQQRNG RKTLTTVQGI
ADDYDKKKLV KAFKKKFACN GTVIEHPEYG EVIQLQGDQR KNICQFLLEV
GIVKEEQLKV HGF.
```



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## FIG. 35E

## PEGen 42 cDNA Sequence

```

GGCGTTGCGA CGTGGACATG TCGGCGTCGT TGGTCCGCGC CACCGTGCGG
GCCGTGAGCA AGAGAAAAC TCAACCCACG CGGGCGGCGC TCACGCTGAC
CCCCTCTGCT GTGAACAAGA TAAAACAAC TCTTAAAGAC AAGCCTGAGC
ATGTGGGTCT GAAAGTGGGT GTGCGGACCA GGGGCTGTAA CGGCCTCTCT
TACAGCCTGG AGTATACAAA GACAAAAGGA GATGCTGATG AAGAAGTTAT
TCAAGACGGA GTCCGAGTGT TCATCGAGAA GAAAGCCCAG CTAACCCTGT
TAGGCACAGA GATGGACTAT GTGGAAGACA AACTGTCCAG TGAGTTTGTG
TTCAACAACC CCAACATCAA GGGAACCTGT GGCTGCGGTG AAAGCTTTAA
CGTCTGAAAG CTGAGGACTG CAAACTCCAG GAGAGCTGGG TCTGCCTTGG
AGCACACCGA AGAAATCATG TGATGTCCCG TGTCGGAAGT TAGTGTGTGG
CTGCCTCGTG GTTGAGAATA AAGTGAAGCA TTGAAAATCA AGCCAGCGTG
TTAGAGTTCC AAAAACATGG TGTCTGTTCT CTGTAAGACA CAAATGGAGA
GAACATGGTG TCTGTTCTCT GGAGGACACA AACTGAGAAA CTGTTGAGTC
CTCTGTCCTG TACAGAAAAC TCCTACCCTG CCCTTACGCT GTAGCCTGCT
CTGTGCTAGA ACCAGCTTCG TGACCATTGC TTTGCTGGGA ATTGAGGAAT
GGGATAACGG GTGTGCACCT GGGTCACAGA ATGGCTTGAG ACTGTCTCCT
GGCCCTGTCT CACCTCAGGC AGGGCAGCTG TGGGAGCAGC AGCTGTGGGA
GCGGTGAGGG GACCTGGTTT CCCTCACCTG TGGCGTGGCC CGTTGCATCT
TTACCACGTG CCTGTTGTCA GATACCTCAT TTGCCAGCCT CCAGCAAGCT
CAGCTATGAG TGCCAGTCTC AGGAGGTAGG GATCACGGGC CTGGTGTCAG
TCTGTCTCTT GGGGCGTGCT TCATGCGGTT TGCTTAGACC TTTCAGTTAG
AAGCGCTTGT GATGAGCAGC CAGGTAGACC TGCTGAGAGC GTGGTTCTCA
GAGCTTCTGC CCAGCCCTCC TCACAGGTCA CAGCAGACAG TGCTGTCTGA
GACACTCGGT GAGGAGACAT CCTGCCTGGC CAGTGCTCTT ACCAGTTTAT
AGACTGCATT AGTTTTCTCT TGAATGGAAG CCTTGTGTAA ACCCTTTTGT
CTGAATGGCC ATCCTGTTTA GAGCTTTGAA CCAGTAGTGT CTTCCCTTCAG
AAGATCTGCA GCAGAGGGGT CCCTCTCAGC ACGGCACCTG GGGGGCAGAA
CATGCACACA CTTACAGTTG CCAGGGTGCA GATGCTCCCT GCTTCCCAGA
GGAAGCTTCT AAGTTTCTTT AATGTGGTCA TCACCAGTTT TTTGAGCCAT
GGTTTTGCTG TATACTACAG GCCAGCCTTG AACCCACAAC AATCCTCCTG
CTTCCACGTT CAGAGGCATG TGCTACCACA CCTGACCTGG ATCCCAAGTT
TCTCTTTAAG TGGTCTTGAT GGACTTGGGT CGGACATCTT AGTGACCTGT
GAATTCTTCT GTGGAGGCTG AGTCTCACGT AGCCGAGTTT AATATCTGTG
CTATTTACTA AAGTATCTGC CACCAAATTG TACCAACTCA TAGTTTTATA
TGAATGTTGA TGAGTCTGTA TCATAAATAG AATTGTTGAT ACATCCTTAA
TTTGTGCAAT ATTGTATGAA GAAGATTGTT ATCAATTAAA ACCACGCCTC
TTTATGATCC TAAAAA AAAA AAAAAA AAAAAA AAAAAA
AAAAAA

```

## PEGen 42 Protein Sequence

```

RCDVDMSASL VRATVRAVSK RKLQPTRAAL TLTPSAVNKI KQLLKDKPEH
VGLKVGVRTR GCNGLSYSLE YTKTKGDADE EVIQDGVRFV IEKKAQLTLL
GTEM DYVEDK LSSEFVFNPN NIKGTCGCGE SFNV

```

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FIG. 35F

## PEGen 45 cDNA Sequence

ACGAGCTGAA GGTCACTTCG CGCACGGGTT GGACCTGGGG CAGGTTGGAG  
GAGTAGGAGT ATGTCATTGG GCGCGAAGAC GGGGTCTGGG GCAAAAAGA  
AGGGAGGCTG GAGAAATCTG GACCCGAGAC GTAGTAAGTA CAACTTGCCA  
AATACATGTT AGAGGAGCAG GGACCACGCT CATCAAAATC CATCATTTGGG  
CTACCTTGGG CTCTCCGCAG TAGCCGAGCT TAACATGATT CTCCACTGCA  
GCTGCCTCTT TGAAGCGGAT CCGTGAAGTA GAAATTTGGA GACGTAAGCT  
GACGTGGAAA TCTATCCCCA TCCTTAGCAG GGAGGTGCTG GTCATGTGAC  
CCGATGTTGA AATTGACAAG CCGCGAGCTA GTCCCGGCTT TTTTTTTTTA  
ACCCCCCTCC CTTTCCTTTT TTCCCCCTCC CCTCCCTCCT CGGCTTCCTT  
TCTTTGTAGC CACCTCAGGG GAAGCAACAG ATCGTCACTC GGTGTTCTCA  
CCGAAAGCAC GTAATCGCCG GTGTAACCTA TGTGCTGGTGG GGGGCCTCCC  
CGCTCGCAGA AAGGCTGGGG TGCGCCCCCA AGCAGCTTTC CTTTGCTCAG  
CTGCATGGTC CTGGTCCACG AGCGCTCTGA GGGCGGCAAG AGAGCGCAAC  
TCCTGACGCC TCCCCCACT CCCCAGTGGG TGAGGGATGC TCTGGGATGG  
GGGTGGCCAG GTGAACGCCC GGAATTGTGT AGCTTCAGGT TCCGGAGTCT  
GTTGTCCGAA GGCTTACGTT CAGCACCTTC TTCGCAGTCC CCCTCCCACA  
GACTTGCTCT GGAAAGCACG TCAGTCTCAG AATCTGGCTG GACCCCATTT  
GGGGCCAGGC TTCGCAGCCA CGATGTGCCG GGCTTCGTGG CTTGTCCGAT  
TTGCACGGTG ACTTGATTAC ACGCTCTCAT TCATGGTCAC TTCCGAAGCG  
CTTTAGTGCC TTCCGTCCCC AAACCGCCAA CAGGCAAAGC GGCTTTCTCT  
CGCGGTTTGT CAATAATCCG CGCTGTCCGG AAGGGCTTCG CCTTACCCGG  
GTTCCACCTT CCCTGTATCT TTCTGCTTAC TTCCTCATCC CACACTCTGT  
CCTTGGAGGA ACCCCTTCTC CTCGCTGCCT GTAGGGGTTT GGAGTGACTC  
CACAGAGCCA GAGGCGCTTC TGCTCACCAG TCCGCAAGCT GCCTGGTCTG  
CTGAAGCTGA CGAATCGGGA AACCATGCAA TTGAGGCGAA CCTTGGGCTG  
CTTTAGAGGC GCTGAGGAGC CTTCTCCTGG GAGGCCCAAG GTCGATTTC  
GCCCACCAGG ATCTGGGGAA GACCCAACTA GGGGTAAGAG CACACCGGAA  
GGCCAAGTCC GAGTTCCAGT CCTAGAAGAG GCGGCTGCGG GCAAGGTTAT  
GACATTGGCC CTGGACACTG GTTTCCCAGG AGCTATTCTT TCTCAAGAAC  
TCCACAGCAC GGGGCTGTCT CCAGAAAATA CTCTTCAACG TTTATTTCTT  
TTAATCGTCA ACCCGCAGCC CTACGGCGGT TAATGCGAGA GGCCAAAAAT  
GTTTGGAGGA AGAAAAACAA AGGCAGGAAG TGGCCGCGGC CTGACGGTGC  
GTGTGTGTCT GTAAAGAAGG GAGGGAGCCG GTTCAATCTC TTCTTTTTTT  
CCCCGAATTT CAAGGTTTAG GCAGACCCCC GTAGGGCCTG GCCGAGGCTC  
ACCCGCGCGA GCATTTGGAG GTGGCCAATG AGTAAGGCTC GTCGGGCTGA  
AAGGCTAAGA AGGAGATTG ATCGGCAGAA CAAACCAAGC CTTTTTGGAG  
GTTTCTTCTG ATTTGGTCCT AAAGGGTATA TGCTAGTGTC CACAGCGGCT  
CCTGTGGCTG CTGTTTTCTT CCTGTCCGAC TAAATGTACC AAGAAGGGAG  
AGAGATTGAG GCACCTTGCG CGCTCCTCTC TCCTTCCGAG GTAGAATATC  
AGAATAAAGT GTATTCAGGT GCCAA

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## FIG. 35G-1

## PEGen 50 cDNA Sequence

A:

```
ATCGGGCTGT ACTAACAGAT TGTTTGTAAG CAGTGACACA GTGATAACTT
CCGTGTTACT TCTTAACCTT ATGTTTCTGC TTTCAGATCT CCCTCCCCTT
CCAGAGGAAG TTAGCGATGC CATAGCTTTA ATGTCTGTTT TAGCTGCAAA
ACTCATTGTT CACTTTCTGT TAGAAAATCT AAAGCAGGTG GTATGCAATT
TCTCTTGATT TGGAATTCTT TAAAGGCAAG TAAATTTGGA ACTCCTGTGT
TGGGGGGTTA ACGGAGGTAG GAACCCAATG GTGTGTCCCT AGGTCGTCCC
CGTTCTCGGA TAGCACAGTC TGCATAGCCA TAGCTCTCAA TTATGTCACT
ACCCTAATCA TCGCAGCCCG GTTCTCACGG ACTCTTTGAA GTCCCAAAAT
GACTTTTGTT TGATCCTGAT TTGGATTTTC AATGGAAAGT AAAAGCTTGG
GGTGAGGAAG CAGCAGCTAA AGCAGGGAGT TGAGCCAGTG AATTGCTGAC
GGAAAGGATT CTGGTCTTGG AGGAGGGGGA CCTGAAGCAG AAGGAAAAGG
GATCCTTCGC TTAAGTTCTT AGGAAAAATC TTGACTCAGA ATCCCAAGAT
TTTTCCCTTC ATCCCAGCCG GGTAAATATT TGGTTTTGTC TTTTAAGTAT
AGCATGAAGC CCGTGGATGA GAGCCATGTG TTGTAGGATT CTCTTCCCTA
TTGGCTCTGA GCTTGTGTCA CCGTATCAGT TTGCTCCCTA CAAAGGGACC
TAGTTTGGAAG AGGATTGGAA GGGCAACTGT TCAGCGGCAA TGGAACACCC
AAACGTGGAC TGGGACAACG GGATTCTGAT AAAGGGAAAT TTCTGGTCTG
GTCCTGGCTG TGTCATAGCT CTTTATGTGT GCATGGAGAG CTCTTGATCC
AAGTAGAATA TGTAACAATA CAGACCAGGA TCTTCCAGTC AGTACTGCTG
GGTGGAAGTG GGCGGGTGAT GGTAGTTGCT AGAAGAATCA TTAAGACAGC
ATCTGCGGTG AATGCGTCCC AAAGCCTCGC GGCATCAGTT TCATCTCTAA
ACCATTAGCT TACAGTTGAT TCCGTTTCCT GGGACAGAGA AACATCCCCA
CGCGAAGTGA CTGTGTTGTG TATTCATAGC ACTGCAAATA AATTCACGCG
CCATGATGAA ACCTTGCAAA TACGCTTTGA CCAAAAAAAAA AAAAAA
```

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## FIG. 35G-2

B:

```

GGGTGTGGGG CAGCTGGGTG GGAGCAGCGT GCAGGCTACC AGCACCAAGT
GGTGTGCCTC TCCGGGGGTG TGTGCAGAAG GCTCCTGGGG AAAACTGCAC
AGGTACCACC CCTAGACAGA AATCGAAAAC CCACTTCTCT CGGTGCCCCA
AGCAATACAA GCATTACTGC ATCCATGGGA GATGCCGCTT CGTGATGGAC
GAACAAACTC CCTCCTGCAT CTGTGAGATA GGCTACTTTG GGGCCCGGTG
TGAGCAGGTG GACCTGTTTT ATCTCCAGCA GGACAGGGGG CAGATCCTGG
TGGTCTGCTT GATAGGCGTC ATGGTGCTGT TCATCATTTT AGTCATTGGC
GTCTTGACC TGCTGTCATC CTCTTCGAA ACATCGCAA AAGAAGAAGG
AAGAGAAAAT GGAAACTTTG AGTAAAGATA AACTCCCAT AAGTGAAGAT
ATTCAAGAGA CCAATATTGC TTAACCTAAT GATTATAAAG TTACCACAAG
CTGATGGCGA GCTCCAAAAG ACCTGACTCA TTTGCAGATG GACAGGACAT
GTCTCAGGAA AACAGCTTGC AGAAATGAAT GTTTAAATAT TGTATTTGCT
TTTTCATTTT ATTTGTAAC GTGTGTTGTT ATTGTTTTTA ATAATGATAT
TTTTGTTACA GTCTGATAGC TGAGAAAAAA ATGACCTGGT TAGGTGACGA
CAATAAGGGA CATTGAATAT AACTTTGTT GCTAGGATTA TTAAACAAAC
AAAATTTGGA AAGAAGTTAG ATTTTAAGAA CTGAGTCATG GTCAGGCAGC
GATGGCACAC ATCTTTAATC CCAGCACTTG GGAGCAGAGG CAGGTAGATC
TCTGGGAGTT TGAGGTCAGC CTGGTCTACA AAGCAAGATC CAGGGTAGCC
AAGGTTATAT AGAGAAACCC TGTCTCACAA AACCAAACCA ACCAATCAAC
CAACAGCAA AACACCTGAG TCGATAAAAG GGCTCCCCAG GTTTATACAC
TTACCGTATG CTAAGAGCTT GAAATATATT GTTTCGTTTT ATCGTTCAGT
AGTCTGTGAG ATTGCATTTT TTCTCATTCC TATATATAAA AAAGTTAAAT
GATTTCCCTT AGATGTAGAG ATAGAGGAAG TTAGCGATGC CATAGCTTT

```

## FIG. 36

PSGen 27-Novel

```

NTCNNCTTNN CNNNGGCTGA TATCNGGCNC TTCNTCCNCG ATCNCAGATA
CNNGCNCACC GGNNNTNTCN GNGGTNATCN TCCNCCATCT CTCNTCCCCG
ACNTGCACTC CGGGTNTNNT ACACNGGACA CTGTATCNNA CAGNAAACCT
NCCCNGGCCC CAGGGATCAC CATNCCTCGN CCCNGCNTGT NTATAANATC
AGGNNTACA TCNANGAACN NACTATCACN GNTCTCTNTT NNCTCAGTGT
NCACCTTCCA CTNCNGAANC TNNTCGCTNC NCCNCNGTTG GGAAAGGCGA
NCNGTNCCGG CNACATGCCG TTTNCGNCNT CTGNNCACNT GGGGATCTNC
TNCAANGNAA TCAATTNGNG TAACCCACGG TTTNCNCAAT CACTACTTCT
CANNCNANGG CCNTTGAANT GTTATCCCAC CACCANGGGG CNANTCGGGA
CCTNACAATT CATCCTCAGC CGGCCCCAGN CTTAAAAAAT TCAAAGGN CN
CTTGCCCGCN TTNTTNCCTT AGCCCCCNC CNGACAACAN CCNANNAACA
ACCCCNNTC TTANGTTGCN NANCCACAG GANNTTGNN TACCGGGTTT
CCCCNGAAAC TNCTCAANGC CNCCGTTCCA ACCCCCGTTA CGAAACCGTN
CCCNTTTCCT TCCGAGNTTG CCTATTAANN CCCCNAAGT TCTNCTTCGT
TNGNTTCCTC CGAAANG

```

- 1 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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(i) APPLICANT: The Trustees of Columbia University in the City of New York

(ii) TITLE OF INVENTION: RECIPROCAL SUBTRACTION DIFFERENTIAL  
DISPLAY

10

(iii) NUMBER OF SEQUENCES: 24

(iv) CORRESPONDENCE ADDRESS:

15

- (A) ADDRESSEE: Cooper & Dunham LLP
- (B) STREET: 1185 Avenue of the Americas
- (C) CITY: New York
- (D) STATE: New York
- (E) COUNTRY: USA
- (F) ZIP: 10036

20

(v) COMPUTER READABLE FORM:

25

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

30

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

35

- (A) NAME: White, John P.
- (B) REGISTRATION NUMBER: 28,678
- (C) REFERENCE/DOCKET NUMBER: 55551-C-PCT/JPW/AKC

(ix) TELECOMMUNICATION INFORMATION:

40

- (A) TELEPHONE: (212) 278-0400
- (B) TELEFAX: (212) 391-0525

## (2) INFORMATION FOR SEQ ID NO:1:

45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 371 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

- 2 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 TAAANCGGTG GTACTGCTGC ACGGTCCTCC GGGTACTGGA AAGACATCCC TTTGTAAGGC 60  
ATTAGCCCAG AACTGACCA TCAGACTGTC AANCAGGTAC CGGTATGGCC AGTTAATTGA 120  
AATAAACAGC CACAGCCTAT TTTCTAAGTG GTNTTCAGAA AGTGGCAAGT TGGTAACTAA 180  
10 GATGTTCCAG AAGATTCANG ACTTGATTGA TGATAANNAA NCTTTGGTGT TTGTCCTGAT 240  
TGATGANGTA AGCACTCANN GGTACTCATT CTTNGTCTGC ATTGCCTCTT GCTATTACTG 300  
15 CCTGATCCCT CTCATTTGGT TCACTGTGTC GCNANCTCTT TTCTATGGAT CTTTCCNAN 360  
CCACCCGTTT C 371

20 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 245 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGACGTAGG GTCTGTTGCG TCAATGGTTA TAGCAAGTGA TGCTCTCTGA TTATTACTGC 60  
TGACAATACT CGGCCAACAA TTCTTGATA GAGTGCTGAT AAATAACTAT GTTACAAAAA 120  
35 GGGGTGGTCC CTGGAGAACA TTACAGGCTT CCCTAGGTAA GTGTGCAGGT CAGGAGACGG 180  
CATATTCAAT CAGATGGCTG ATAGTTCTCC GTGGTTATGC ACCGGCTCCA GCTTGCCTAC 240  
40 GTCAC 245

(2) INFORMATION FOR SEQ ID NO:3:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 178 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant

- 3 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCAGCATGAT GAATTTAATG CAACAGTCAT AGCAGGGCAA GGGGAGAGAA AGGCAGATGG 60  
ACTATCTGCA TCATCAAGCG AGGGCTTGTG TCGGCGGCTA TGTGCAGAGA CGAGCAGGGC 120  
10 GAGGCACTTA AAAGCTGCTN GATGAAAATC CACCCAGGAG AANTCTGGGC CTACGTCA 178

(2) INFORMATION FOR SEQ ID NO:4:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 191 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25

TGACGTAGGC CCAGACTTCT CCTGGGTGGA TTTTCATCCA GCAGCTTTTA AGTGCCTCGC 60  
CCTGCTCGTC TCTGCACATA GCCGCCGACA CAAGCCCTCG CTTGATGATG CAGATAGTCC 120  
30 ATCTGCCTTT CTCTCCCCTT GCCCTGCTAT GACTGTTGCA TTAAATTCAT CATGCTGCCA 180  
AAAAAAAAA A 191

(2) INFORMATION FOR SEQ ID NO:5:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 124 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

45

GCCATAAATA CACTTTATTT CATTGAAAT GCATAATCAC ACTGGGAGCA CTCCCTTTGG 60  
AGCACTCCTC TAGCAGCAGG TCCGAAGTGC TCCAGCATCG TCAGCTGGCT CCAACACCTA 120

CGTC

124

## (2) INFORMATION FOR SEQ ID NO:6:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 61 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

15 TTTTTTTTTT TTTGGAAACA GAATAAGTG CTTTATTCTC TGGCTGGCTC TCCTACGTCA 60  
C 61

## (2) INFORMATION FOR SEQ ID NO:7:

20

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 216 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30

TCGGCGATAG CATTGGAGCA AGTCTTATCA GCAAGCAATG TTTTCAGTTA TGTTCAAAG 60  
TTAAGAATGG GTTTAACTT GCTGAACGTA AAGATTGACC CTCAAGTCAC TGTAGCTTTA 120  
35 GTACTTGCTT ATTGTATTAG TTTANATGCT AGCACCGCAT GTGCTCTGCA TATTCTGGTT 180  
TTATTAAAT AAAAAGTTGA ACTGCAAAAA AAAAAA 216

## (2) INFORMATION FOR SEQ ID NO:8:

40

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 334 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA



- 5 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TNGCCAGGCT ATGTCTCAGA 60  
 5 CTTTATTATT ATTATTATTA TTATTATTAT TATAAATAAA ACATGTNCTT TCAATTAGGT 120  
 TACAANAGTA TTTATCTCCA TAACGCTTCT TCATACATCC TTAGTTTGGG ATTAAAGTAC 180  
 CATCCACCCC AACTCAAACGT GTAACCCCCA GTAATCCCCT CTAACGTGGA AATTTCTGGT 240  
 10 TTAACAACCTC AGTTAACTGC CCCACAAACA GTGGGAGGCC GCTCTTGCAT GGCTATGCCA 300  
 CGTAACCCCTT CACTGCTTCA CTTCTTCGCT GGCT 334

15 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 136 base pairs  
 (B) TYPE: nucleic acid  
 20 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACCGCTTGT ACCATCCAAC TTGCTTTGTC TTCTGCAGAG AGGAGGCTAA AGCCCTTGAG 60  
 CTGGCTGGCA CTGTACTCAG GCCGGAAGCC CAGCTCGTCC CGGTTCTTGA CAAAGCAAGT 120  
 30 TGGATGGTAC AAGCGG 136

(2) INFORMATION FOR SEQ ID NO:10:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 316 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: not relevant  
 40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

45 TGCCGAGCTG GGTATTGTGA CGGTTGATAA TGGCGGCATC ATGTTGCCAG GTACCGGGTA 60  
 AGCAGACCTC AGAGCACAGC TTATTGTCCA GTGCTTTCAC GCTCGCGACG TCAAAGTCAT 120

- 6 -

TGTTATTGTC ACACTCCATG CCTAGAAATG CGCATGTCCT CTGGCCATCT TCTTGACAG 180  
GGGATCTGTC CTCTTCCTCC ATGATATCAT TTCCCTCTGC ATCCTGCTCT CCAGCTGGAA 240  
5 GGCCAGCAAA ATTGCTGTCT GGGGACTCTG CTGGGGTCTC CTCCTCTTCT GAAGGGGCCC 300  
TGCTAGCAGC TCGGCA 316

## (2) INFORMATION FOR SEQ ID NO:11:

10

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 337 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
15 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

20

AGGGGTCTTG ATGGACTTGG GTCGGACATC TTAGTGACCT GTGAATTCTT CTGTGGAGGC 60  
TGAGTCTCAC GTAGCCGAGT TTAATATCTG TGCTATTTAC TAAAGTATCT GCCACCAAAT 120  
25 TGTACCAACT CATAGTTTTA TATGAATGTT GATGAGTCTG TATCATAAAT AGAATTGTTG 180  
ATACATCCTT AATTGTGCA ATATTGTATG AAGAAGATTG TTATCAATTA AAACCACGCC 240  
TCTTTATGAT CCTNNNAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 300  
30 AACCNCCTCA AATCCATNGG TTCTAACCCA AAACCCCT 337

## (2) INFORMATION FOR SEQ ID NO:12:

35

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
40 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

45

TTTTTTTTTT CATACACCAT CAAACCAATT TTATTTCTAT AGCAACGTTT CTCACGTCTG 60  
AACCTGAGAA TAAGTCACCA GCTCTTGACA GTAAACATGG GCCCTATCAA ATTATATTAG 120

- 7 -

ACTCCTCAGT GTCCCGCCAT GTGGCCTTGC ACCAAATCAA TTAGTTTGAG GGCCAAAATC 180  
CTGTTGGGTT TCAAATAAAG TGTCAGGTCA TAAGGAGGGG GAGGGACTCA ATTCATGGGA 240  
5 ACATTTTAC CTGTTCAAAT AGATAAACTG AATTGCCCTA TCTGTGGTCA CCTGGATCCA 300  
AGACCCT 307

## (2) INFORMATION FOR SEQ ID NO:13:

10

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 296 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
15 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

20

CCCTGACGAT AAATGGTAAG GAACTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTNC 60  
GAAATAAACA AACACAGCTT ATTATTTGGG GGAACATTAA NTTCTATAAN TGAACACAAA 120  
25 ANAAAATTAA NANTTAATGG GGGGGTANAA GGGACTTTGA ATCTATCTGG TATCATGACA 180  
TTGAAGCANA NACCTGANTG ACCAGAAAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA 240  
GAGAGGTTTC ATATGAGCTA GTGTTACAGG CTTTATTAGT CTATTAGTCA GGGACC 296

30

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 319 base pairs  
35 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

40

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AATCGGGCTG GATGGGTGTA TCCGGCACTG TTTCGTAGCG GCAGCAACTG GGTGCTTCTA 60  
45 TCTGAAAGCG GGCTTCACAA AAATACTGC GCCACCCGAC TCGCTGCGGC ATCGCCCGGT 120  
GGCGAGTACC GTATCGCCTT TCCTGGTGCA GAAGAAGTGT TTACAGGAGG CGGTCATTTA 180

- 8 -

CCGCAATCTG ATTCTGTTTT TTATTCTCCC TGGCGGGTGA TCGCGATCGG CAGTTTGAAA 240  
ACGATCGTTG AATCCACGCT CGGGAATGAT GTGGCTTCGC CGCCAACGCT TACTGACATT 300  
5 TCATTGTAC AGCCCGATT 319

## (2) INFORMATION FOR SEQ ID NO:15:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 287 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

20 GCCGAGCTGT GTAAACCAT CTATCCTCTG GCAGATCTAC TTGCCAGGCC ACTCCCAGGG 60  
GGGGTAGACC CTCTAAAGCT TGAGATTAT CTTACAGATG AAGACTTCGA GTTGCACCTC 120  
GACATGACCA GAGATGAATT CAACGCACTG CCCACCTGGA AGCAAATGAA CCTGAAGAAA 180  
25 GCGAAAGGCC TGTCTGAGG GTGAGATGAC AGCCACAGAG AGGTCACCTGC CACTAGACCA 240  
GAAAGTGGAT GGAGATATAT ATTTGGACTG GTGTTTTTTT CTGTCAG 287

30

## (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 344 base pairs  
35 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATCGGGCTGC AGATTGGAGA CAAGATCATG CAGGTGAACG GCTGGGACAT GACCATGGTC 60  
45 ACTCATGACC AGGCTCGGAA GCGGCTCACC AAACGTTCGG AGGAAGTGGT CCGCCTGCTG 120  
GTGACTCGGC AGTCTCTGCA GAAGCCGTA CAGCAGTCCA TGCTGTCATA GCTGTAGTCA 180

- 9 -

GCCTAGACTT CTGCCCCTG ACCTTTTNGG GCACTGAGAA CACATCCACG CTCTGTCTGT 240  
ATCTAGTTCT GGCTTCTGCT GTGTGCTANG CCCCAGCTCT GAGGAGTAAC AGCTGATCCC 300  
5 AAAGGTCCAA GCCAACCTTC TTACCCCTCA GCCCCCANCC CGAT 344

## (2) INFORMATION FOR SEQ ID NO:17:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 300 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

20 TTTTTTTTTT TTTGGGCAAC TATGTATTTA TTGTGTTTGG AAGGCAGAGT GAGGGAGGAG 60  
ACCCCAGCAG GAAGAAGACT GGGTGCAGTC TAGAGTTCCT AGTCAAGAGT AGGAAGGTTT 120  
CTGTTATACC CATCATAGAA CGAGAGAGGG GGCTCAATAG ATCATCCCCT TTGTCTCTCC 180  
25 ACGGGGCTTC TTGAGCTTCT CAAAGTTCTT CAGGATGATG TCATATAACA CAGCATAAGC 240  
GTTACGGATC TCCATGACCA TCAGCCGGAT CTCCTGGTAT TCCGCCTCGT CCAGCTCGGC 300

30 (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 461 base pairs  
35 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

45 AANATCTGCT TAAAAGTTCT TTAATTTGTA CCATTTCTTC AAATAAAGAA TTTTGGTACA 60  
AATTAAGAA CTTTAAAGCA GATGTTTTGG TGCAACTAAT AGAAAAGATA AAGGCAGCCT 120  
GACATGCATG CACTGCCTCA GTGACCAGTA AAGTCACATG NCCTTGGGAC GTCAGCTTAG 180

-10-

NTTTATCACN GTGTCCCAGG GGTGCTTGTC AAAGAGATAT TCTGCCATGC CAGATTCAGG 240  
GGCTCCCATC TTGCGTAAGT TGGTCACGTG GTCACCCAGT TCTTTAATGG ATTTACCTG 300  
5 CTCATTCAGG TAATGCGTCT CAATGAAGTC ACATAAGTGG GGATCATTCT TGTCAGTAGC 360  
CAGTTTGTGA AGTTCCAGTA GTGACTGATT CACACTCTTT TCCAAGTGCA GTGCACACTC 420  
CATTGCATTC AGCCCGCTCT CCCAGTCATC ACGGTCACNT A 461  
10

## (2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:  
15 (A) LENGTH: 280 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

25 TGACGTAGGG CCGAGAGCAA CAAGCACAGA ACTCCTTCTC CAGTTTCACC CTGATGAAGT 60  
TGAGGCACTC TTCTGCACTG GGAGGGGCCA GCCTGGGGGC CAGGCACATT GGACACCACC 120  
TTCCCATGGA CTACAGCGTC AATGCCATTG CCTTCTATTC CTATACCTTC TAGGGGCTGC 180  
30 CCCTCTTCCC ATTCAGCCAA CACTGAGTGT TGGGAGATT CTCTTTTTA AAAACACATG 240  
AGAAAATAAA TGCACTTTAC TCCCTCCCCA AAAAAAAAAA 280

35

## (2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
40 (A) LENGTH: 177 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

- 11 -

GTAGGCAATA AAATGTTTTT AGAGGTGCGA AAAAGCTTTT GTTTTCTTAA ACCATTCTTA 60  
GTCTCTGCCA CACTTGACAC TCCGTCAAAG TGAGAAGCGA ACTAAAGACC AACTGCGGTG 120  
5 GAAAATATTA TGTTTATGTA ATAAAAAAAA ATCATGTAAC TGCAAAAAAAAA AAAAAA 177

## (2) INFORMATION FOR SEQ ID NO:21:

## 10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 633 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

15

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

20

TGCCGAGCTG AAAACATACA TCCGCACCGG GTTGAATAG CTGGCCCTCC GTCCCCGGGC 60  
ATACTCTTTG GATAAGAACC CCGGCCTTGT TACCAGGTAC CGGAGTGAGC TGAAAAATTT 120  
25 ACCGTCGAAA TGGGTGATGT CCTGGAAAAA ATGGTTCACC AGCTGCCAGG CAGATTCTTT 180  
GGGTTCACA TTTCTCTGCC CACAGATGTG GCAGAAGCGG TCAAGTAATG CAGCATTACA 240  
ATTGAGGCAG ATCTTTTCTT TTCTTTCCTT GGAGTGGCTC AACCAGCGAT TTTGGTTAAA 300  
30 AATAATCAAA AAAGCGACGG CAAACTTTT GTTATATTCC CGCCTGTGGC ATTTGAACTG 360  
TGCCCGGCAA CCGAATAACT TTAAATTTTG AAAATAAAAT GCATACTAGA TTTTTCAGCG 420  
35 TTGCCTCCTG GCCATTGCTT CAGGCGCCNG CACAGCGTCA GCCCAGTTT ACCACNANGA 480  
ATATCCTAAG CGTTGAAACA GGGCACAGCC GAAAAAACN CTGGCNACAA AAAANATCCG 540  
GACATCCTTT TTCCAATTTT GAAACCGAAN GCNCGCAAAC NAAGGTTCTT CGGGAAAAAA 600  
40 AATCGCCAAA ATACNCGANA TCAAACTNTC CAA 633

## (2) INFORMATION FOR SEQ ID NO:22:

45

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 213 base pairs  
(B) TYPE: nucleic acid

-12-

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

10 TGCCGAGCTG GGGGGAGTTC CAGGAATTG TGGACTATTT CCAGGAGGAA TTGAGGAATC 60  
TAGAAGTAAT AAGAACTTCA CAAGTAGAAC AACAGAGTTA ATTGACCTCT ATCCTTAAGA 120  
GTTACCAGAG AATTATTAAA AACTAAAGA ACAATCAAAG CCTGGTCCTG TGCCACCACC 180  
15 CAAAAACATG TATAGCCTAT GTGCAGCTCG GCA 213

(2) INFORMATION FOR SEQ ID NO:23:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 679 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

30

CTCANAGGGC NNNTTNGNGG NCNTCATGCN CCAGGNTCCN NCCCCANAN GANCNNCCNG 60  
GTAAACTACA CNGGAGTACT TAAGTGGACA NNCCACATGC GANGGNAAG GGGATCACCN 120  
35 TCNCTCCTNC AGNCTNTNCG TGNCTCTCCT GTNCNTNCAC TGCCNCANAA NGGANGCNCN 180  
NNCTCCTATC TGTNTACAGN AACNTNGCN CTNNTCTAA GCTCNCCCAC TNTGTGGAAG 240  
GGCNATGTGT GCGTGCCTCT CCCCTATCAC GGCNGTTTGC NAAANGGGGA TGTNCTGCNC 300  
40 GGCGATGAAG TTNGGTCCT CCATGTTTCC CAGTCCNACC TGTTAGACNA AGNATTGNAN 360  
TGTGATACGA CTCNCTGTAA GGGGANTNGC GGACCCAGTA TGTTTGCCCC NACNNCCACT 420  
45 TCTTTAAATG GTGGCTAACG GCGCTTCCTA GNATAAACAC TATTGGTCCC CCCCTCTGCA 480  
GNACCCNTTA CTTCCGNANA AAAATTGTTG TCNTGATCCG CGACAACCAC ACCGTCTGTN 540



-13-

GNTTTTAGTT GCAACNCNNA TCNCTCCAAA AAAGTTTCAG AAATCTTCAT TTTCCCNCGT 600  
 TGAGCCCNCTG ACAAACCCCT NAGGATTTGT CGAATGTAAA GTCTCCNGAT CTTCAATAAA 660  
 5 NNTCCAAAAG NCTANCGAT 679

## (2) INFORMATION FOR SEQ ID NO:24:

## 10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 717 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

15

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

20

NTCNNCTTNN CNNNGGCTGA TATCNGGCNC TTCNTCCNCG ATCNCAGATA CNNGCNCACC 60

GGNNNTNTCN GNGGTNATCN TCCNCCATCT CTCNTCCCCG ACNTGCACTC CGGGTNTNNT 120

25

ACACNGGACA CTGTATCNNA CAGNAAACCT NCCCNGGCC CAGGGATCAC CATNCCTCGN 180

CCCNGCNTGT NTATAANATC AGGNNNTACA TCNANGAACN NACTATCACN GNTCTCTNTT 240

NNCTCAGTGT NCACCTTCCA CTNCNGAANC TNNTCGCTNC NCCNCGTTG GGAAAGGCGA 300

30

NCNGTNCCGG CNACATGCCG TTTNCGNCNT CTGNNCACNT GGGGATCTNC TNCAANGNAA 360

TCAATTNGNG TAACCCACGG TTTNCNCAAT CACTACTTCT CANNCNANGG CCNTTGAANT 420

35

GTTATCCAC CACCANGGGG CNANTCGGGA CCTNACAATT CATCCTCAGC CGGCCCCAGN 480

CTTAAAAAAT TCAAAGGNCN CTTGCCCGCN TTNTTNCCTT AGCCCGCCNC CNGACAACAN 540

CCNANNAACA ACCCCCNNTC TTANGTTGCN NANCCACAG GANNTTGNA TACCGGGTTT 600

40

CCCCNGAAAC TNCTCAANGC CNCCGTTCCA ACCCCCGTTA CGAAACCGTN CCCNTTTCCT 660

TCCGAGNTTG CCTATTAANN CCCCCNAAGT TCTNCTTCGT TNGNTTCCTC CGAAANG 717

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/04323

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 21/02; C12Q 1/68; C12N 15/11

US CL : 530/350; 536/23.1; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,981,783 A (AUGENLICHT) 01 January 1991, col. 2, lines 40-64.	19
A	US 5,599,672 A (LIANG et al.) 04 February 1997, see entire document.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 JUNE 1999

Date of mailing of the international search report

15 JUN 1999

Name and mailing address of the ISA/US  
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Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/04323

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 20-40  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
The Computer Readable Form (CRF) of the Sequence Listing as filed does not comply with 37 CFR § 1.821-1.824.  
As such, claims 20-40 could only be searched in part, by word searching.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

